Studies of localization and expression of Angiopoietin in the testis

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Content

Abstract.....i

摘 要.....iii

Abbreviations.....v

Acknowledgement.....x

Chapter 1 Introduction

1.1 General review of angiogenesis.....1

1.1.1 Angiogenesis in development and growth.....1

1.1.2 The process of angiogenesis.....2

1.1.3 Types of factors controlling angiogenesis.....3

1.2 Roles of VEGF and its receptors in the regulation of angiogenesis.....6

1.2.1 VEGF.....6

1.2.2 VEGF receptors.....8

1.2.3 Regulation of VEGF expression by hypoxia and nitric oxide.....10

1.2.4 Signal transduction mechanisms of VEGFR-1 and VEGFR-2...12

1.2.5 Anti-apoptotic effect of VEGF on endothelial cells as a result of signal transduction of VEGFR-2.....14

1.3 Angiopoietins.....15

1.3.1 Angiopoietin 1 (Ang-1).....16

1.3.2 Angiopoietin 2 (Ang-2).....19

1.3.3 Angiopoietins 3 and 4 (Ang-3 and Ang-4).....24

1.4 Interaction among VEGF, angiopoietin and Tie in the maintenance of vasculature.....25

1.5 Tyrosine kinase with immunoglobulin and EGF factor homology domains – Tie 1 and Tie 2.....28

1.6 Angiopoietin expression in female reproductive tissues (ovary).....33

1.7 Testicular angiogenesis.....37

1.8 Aims of the present study.....38

Chapter 2 Materials and methods

2.1 Preparation of primary cells from rat testes.....40

2.1.1 Sertoli cell preparation.....40

2.1.2 Germ cell preparation.....41

2.1.3 Interstitial cell and Leydig cell preparation.....43

2.2 Cell cultures....45
2.2.1 Reagents and cell lines....45
2.2.2 Cell lines of mouse TM3 Leydig cells and TM4 Sertoli cells....45
2.2.3 Mouse MLTC-1 Leydig tumour cells....46
2.2.4 Rat R2C Leydig tumour cells....46
2.2.5 Rat LC540 Leydig tumour cells....47
2.2.6 Rat C6 glioma cells....47
2.3 Analyses of Angiopoietin 1, Angiopoietin 2, Angiopoietin 3, Tie 1 receptor, and Tie 2 receptor mRNA in testicular cell lines and testicular tissues....48
2.3.1 Extraction of total RNA from testicular cell lines and testicular tissues....48
2.3.2 Quantitation of total RNA....50
2.3.3 First strand cDNA synthesis by reverse transcription (RT)....51
2.3.4 Normalization of the amounts of cDNA used in polymerase chain reaction (PCR)....52
2.3.5 Polymerase chain reaction (PCR)....53
2.3.6 Purification of PCR products....65
2.3.7 Confirmation of PCR product authenticity by automated DNA sequencing....66
2.4 Western blot analysis....68
2.4.1 Preparation of cell lysates from primary testicular cells and testicular cell lines....68
2.4.2 Preparation of mouse testicular tissue and adult rat testicular tissue lysates....68
2.4.3 Determination of protein concentration....69
2.4.4 Reagents for Western blot analysis....70
2.4.5 Preparation of protein samples and markers for Western blot analysis....71
2.4.6 Sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE)....72
2.4.7 Transfer of proteins to membrane....74
2.4.8 Blocking of the membrane....74
2.4.9 Immunoblotting....75
2.5 Immunohistochemical staining for Ang-1, Ang-2, Ang-3, Tie 1 and Tie 2 in rat testes....78

Chapter 3 Results

3.1 Expression of Ang-1 and Ang-1 alternatively spliced transcripts in the testis and other testicular cell types.....81

3.1.1 Detection of Ang-1 expression in the testis and and testicular cell types by nested PCR.....81

3.1.2 Detection of Ang-1 expression in testicular cell lines by nested PCR.....82

3.1.3 Sequence analysis of Ang-1 transcript amplified from adult rat testis.....84

3.1.4 Detection of alternatively spliced species of Ang-1 mRNA in the testis and other testicular cell lines.....87

3.2 Expression of Ang-2 and Ang-2 isoforms in the testis and various testicular cell types.....94

3.2.1 Detection of Ang-2 expression in the testis and testicular cell types by nested PCR.....94

3.2.2 Detection of Ang-2 expression in testicular cell lines by nested PCR.....96

3.2.3 Sequence analysis of Ang-2 transcript amplified from adult rat testis.....98

3.2.4 Detection of the expression of Ang-2 isoforms in adult rat testis.....99

3.3 Expression of Ang-3 in the testis and testicular cell types.....103

3.3.1 Detection of Ang-3 expression in the testis and primary testicular cells by RT-PCR.....103

3.3.2 Detection of Ang-3 expression in testicular cell lines by RT-PCR.....105

3.3.3 Sequence analysis of Ang-3 transcripts amplified from TM4 mouse Sertoli cells and adult rat testis.....105

3.4 Expression of Tie 1 and Tie 2 in the testis and testicular blood vessel.....110

3.4.1 Detection of Tie 1 and Tie 2 expression in the testis and rat testicular blood vessel by RT-PCR.....110

3.4.2 Sequence analysis of Tie 1 transcripts amplified from adult rat testis and rat testicular blood vessel.....113

3.4.3 Sequence analysis of Tie 2 transcript amplified from rat testicular blood vessel.....113

3.5 Western blot analysis of Ang-1 and Ang-2 expression in testicular tissues, primary testicular cells and cell lines.....116

3.6 Localization of Ang-1, Ang-2, Ang-4, Tie 1 and Tie 2 proteins in adult rat testis by immunohistochemistry..... 122
3.7 Comparison of angiopoietin expression patterns in testis using RT-PCR, Western immunoblotting and immunohistochemistry..... 128
3.8 Comparison of Tie 1 and Tie 2 expression patterns in testis using RT-PCR and immunohistochemistry..... 128

Chapter 4 Discussion
4.1 Expression of Ang-1 mRNA and protein in adult rat testis, mouse testis, rat testicular blood vessel, primary testicular cells and testicular cell lines..... 131
4.2 Expression of Ang-2 mRNA and protein in adult rat testis, mouse testis, rat testicular blood vessel, primary testicular cells and testicular cell lines..... 136
4.3 Expression of Ang-3 mRNA and protein in adult rat testis, mouse testis, rat testicular blood vessel, primary testicular cells and testicular cell lines..... 141
4.4 Expression of Tie 1 and Tie 2 mRNAs and proteins in adult rat testis and rat testicular blood vessel..... 143
4.5 Conclusion..... 145
4.6 Future work..... 146

Chapter 5 References..... 149

Abstract

The possible involvement of angiopoietins and two endothelial cell-specific receptor tyrosine kinases (Tie 1 and 2) in the control of testicular angiogenesis has not been explored in the past. To set the stage for such an investigation, the present study examined the expression and cellular localization of angiopoietins and Tie in rodent testes using reverse transcription-polymerase chain reaction (RT-PCR), Western immunoblotting and immunohistochemistry.

Using nested PCR, Ang-1 expression was demonstrated in adult rat and mouse testes, and rat testicular blood vessels (TBV). In the study of primary testicular cells and cell lines, Ang-1 mRNA expression was again pinpointed to Leydig cells and Sertoli cells. Western immunoblotting confirmed the presence of Ang-1 protein in adult rat testes, TBV, primary Sertoli cells and interstitial cells, and all testicular cell lines (except LC540). Both the non-glycosylated (58 kDa) and glycosylated (72 kDa) form of Ang-1 were found, but the former appeared more abundant. Immunostaining (with another antibody) showed positive Ang-1 immunoreactivity in vascular smooth muscle cells of rat testes. No alternative spliced variants of Ang-1 mRNA could be identified in any testicular cells or tissues.

For Ang-2, the use of nested PCR demonstrated its mRNA expression in adult rat testes, rat TBV, and primary rat Leydig cells, Sertoli cells and germ cells. In testicular cell lines, Ang-2 transcripts were found in TM3 Leydig cells and TM4 Sertoli cells, again suggesting a major source of testicular Ang-2 from these two cell types. Western immunoblotting with a specific antibody confirmed the presence of Ang-2 protein in adult rat (and mouse) testes, interstitial cells, Sertoli

cells and TBV. Both the non-glycosylated (55 kDa) and glycosylated (68 kDa) form of Ang-2 were found. For testicular cell lines, results obtained using an antibody that cross-reacts with both Ang-1 and –2 indicated that in Leydig tumour cell lines (i.e. MLTC-1, LC540, R2C), the non-glycosylated form of Ang-2 was apparently present though Ang-2 mRNA could be demonstrated. In line with the Ang2- mRNA expression in rat TBV, specific Ang-2 immunostaining was localized to endothelial cells. The use of RT-PCR however failed to demonstrate the presence of Ang-2 isoform in adult rat testes.

Using RT-PCR, Ang-3 mRNA expression was found in adult rat testes, rat TBV, primary rat Leydig cells, Sertoli cells and germ cells, mouse testes, rat (LC540 and R2C) and mouse (TM3 and MLTC-1) Leydig cell lines, and TM4 mouse Sertoli cells. In line with the above observation, Ang-3 immunostaining was localized to vascular smooth muscle cells and testicular interstitial cells.

The presence of Tie 1 and 2 transcripts in adult rat testes and TBV was demonstrated using RT-PCR. Their association with TBV correlated well with their immunolocalization in endothelial cells.

In conclusion, this study has demonstrated the presence of the full complement of angiopoietin-Tie 2 system in the testis. It is likely that through its interaction with VEGF and VEGF receptors which are also found in the testis, they play an important role in determining the stability of the testicular vasculature or directing its proliferation (i.e. angiogenesis) or regression (i.e. through apoptosis).

摘要

過去的研究沒有勘探血管形成素 (Angiopoietin, Ang) 和酪氨酸激酶(具有類似免疫球蛋白及表皮生長因子同種性的區域)一型及二型(tyrosine kinase with immunoglobulin-like and epidermal growth factor homology domains, Tie 1 and Tie 2) 在睪丸血管生成的調節機制中所扮演的角色。本項研究藉著逆轉酶-聚合酶連鎖反應 (reverse transcriptase-polymerase chain reaction, RT-PCR)、韋斯頓免疫印跡術 (Western immunoblotting)及免疫組織化學 (immunohistochemistry) 的技術，去考察 Angiopoietins 和 Tie 之表達 (expression) 及細胞定位 (localization)。

巢聚合酶連鎖反應 (nested PCR) 的實驗結果顯示血管形成素一型 (Ang-1) 在成年大白鼠睪丸、小白鼠睪丸及大白鼠睪丸血管 (rat testicular blood vessel, TBV) 內表達。本研究利用初級睪丸細胞 (primary testicular cells) 及細胞株 (cell lines) 來顯示 Ang-1 的信使核糖核酸 (mRNA) 在萊迪希氏細胞 (Leydig cells) 及謝爾托立氏細胞 (Sertoli cells) 內表達。韋斯頓免疫印跡術確定 Ang-1 蛋白質存在於成年大白鼠睪丸、TBV、初級謝爾托立氏細胞、初級間質細胞及所有睪丸細胞株(除了 LC540) 內。兩種 Ang-1 的非被糖基化類型 (non-glycosylated) (58 千道爾頓 kDa) 及被糖基化類型 (glycosylated) (72 千道爾頓 kDa) 被發現，但前者的數量似乎較多。免疫組織化學的實驗 (採用另一種抗體) 顯示 Ang-1 的免疫反應力存在於大白鼠的睪丸血管平滑肌內。沒有 Ang-1 替代剪接變異體 (variants) 的 mRNA 在睪丸細胞及組織內被發現。

至於血管形成素二型 (Ang-2), nested PCR 的實驗結果闡述它的mRNA在成年大白鼠睪丸、大白鼠的 TBV、及初級大白鼠的萊迪希氏細胞、謝爾托立氏細胞和生殖細胞 (germ cells) 內表達。在睪丸細胞株中, Ang-2 的轉錄本 (transcripts) 在 TM3 萊迪希氏細胞和 TM4 謝爾托立氏細胞內被發現，這樣便能說明這兩種細胞就是睪丸的 Ang-2 的主要來源。韋斯頓免疫印跡術採用特定的抗體來確定 Ang-2 蛋白質存在於成年大白鼠(和小白鼠) 睪丸、間質細胞、謝爾托立氏細胞及 TBV 內。Ang-2 的非被糖基化類型 (55 千道爾

頓 kDa) 及被糖基化類型 (68 千道爾頓 kDa) 亦被發現。至於睪丸細胞株方面，利用同時與 Ang-1 和 Ang-2 有交叉反應的抗體的實驗結果指出雖然 Ang-2 mRNA 不存在於萊迪希氏腫瘤細胞株內 (即 MLTC-1, LC540, R2C), 但 Ang-2 蛋白質顯然地存在於這些細胞株內。除了 Ang-2 mRNA 在大白鼠的 TBV 內表達外，特定的 Ang-2 免疫染色被定位於內皮細胞內。RT-PCR 未能闡述 Ang-2 的等型 (isoforms) 存在於成年大白鼠睪丸內。

藉著 RT-PCR, 血管形成素三型 (Ang-3) 在成年大白鼠睪丸、大白鼠的 TBV、初級大白鼠萊迪希氏細胞、謝爾托立氏細胞和生殖細胞、小白鼠睪丸、大白鼠 (LC540 和 R2C) 、小白鼠 (TM3 和 MLTC-1) 萊迪希氏細胞株及 TM4 小白鼠謝爾托立氏細胞株。除了以上的觀察外，Ang-3 免疫染色被定位於血管平滑肌及睪丸間質細胞內。

RT-PCR 的實驗結果闡述 Tie 1 和 Tie 2 的 transcripts 在成年大白鼠睪丸和 TBV 內存在。它們與 TBV 的聯繫與它們的免疫定位於內皮細胞內非常吻合。

總括而言，本項研究闡明 angiopoietin-Tie 2 系統內的所有補體存在於睪丸內。可能透過它與在睪丸存在的血管內皮生長素 (vascular endothelial factor, VEGF) 和 VEGF 的受體 (receptors) 的相互作用, 它們在確定睪丸血管系統的穩定性或使它進行增殖 (即血管生成過程) 或退化 (即透過衰老過程) 具有重要的作用。

Abbreviations

A	Absorbance
AEC	3-Amino-9-Ethylcarbazole
AFGF	Acidic Fibroblast Growth Factor
Akt	Antiapoptotic serine-threonine Kinase
Ang	Angiopoietin
APES	3-Aminopropyltriethoxysilane
APS	Ammonium Persulphate Solution
BAEC	Bovine Aortic Endothelial Cells
BFGF	Basic Fibroblast Growth Factor
BREC	Bovine Retinal Endothelial Cells
BSA	Bovine Serum Albumin
CDI	Cartilage-Derived Inhibitor
CE-LIF	Capillary Electrophoresis with Laser-Induced Fluorescence Detector
c-Ets 1	c-Erythroblast Transformation Specific Domain 1
CL	Corpora Lutea
DEPC	Diethylpyrocarbonate
DMEM/F-12	Dulbecco's Modified Eagle Medium: Ham's F12 medium
DNase	Deoxyribonuclease

DNTPs	Deoxynucleotidetriphosphates
DTT	Dithiothreitol
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
ENOS	Endothelial Nitric Oxide Synthase
FBS	Fetal Bovine Serum
FDs	Fibrinogen-like Domains
FN	Fibronectin
Flk-1	Fetal Liver Kinase-1
Flt-1	fms-like Tyrosine kinase-1
Flt-4	fms-like Tyrosine kinase-4
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
G-CSF	Granulocyte Colony-Stimulating Factor
Grb	Growth factor Receptor-Bound protein
HBSS	Hanks' Balanced Salt Solution
hCG	Human Chorionic Gonadotropin
HGF	Hepatocyte Growth Factor
HIF-1	Hypoxia-Inducible Factor-1
HRP	Horse Radish Peroxidase

HUVEC	Human Umbilical Vein Endothelial Cells
HUVSMC	Human Umbilical Vascular Smooth Muscle Cells
IL-3	Interleukin-3
IL-8	Interleukin-8
IP-10	Interferon Inducible Protein-10
IRES	Internal Ribosomal Entry Site
kDa	kiloDalton
KDR	Kinase insert Domain-containing Receptor
LH	Luteinizing Hormone
LHS	Left-Hand Side
MAPK	Mitogen-Activated Protein Kinase
MMP	Matrix Metalloproteinase
NOS	Nitric Oxide Synthase
Oligo-dT	Oligodeoxythymidine
PBS	Phosphate-Buffered Saline
PBST	Phosphate Buffer Saline Tween-20
PCR	Polymerase Chain Reaction
PD-ECGF	Platelet-Derived Endothelial Cell Growth Factor
PDGF	Placental-Derived Growth Factor

PDGF-BB	Platelet-Derived Growth Factor-BB
PF4	Platelet Factor-4
PGF	Placental Growth Factor
PI 3'-kinase	Phosphatidylinositol 3'-Kinase
PMSF	Phenylmethylsulfonyl Fluoride
PRP	Proliferin-Related Protein
PTN	Pleiotrophin
PVDF	Polyvinylidene Difluoride Membrane
RA	Rheumatoid Arthritis
rhCG	Recombinant Human Chorionic Gonadotrophins
RHS	Right-Hand Side
RT	Reverse Transcriptase
RTK	Receptor Tyrosine Kinase
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
SD	Sprague Dawley
SDS	Sodium Dodecyl-Sulphate
SDS-PAGE	Sodium Dodecyl-Sulphate-Polyacrylamide Gel Electrophoresis
SF	Scatter Factor
Shc	Src Homology 2 domain-Containing

SH2	Src Homology domain 2
SHP2	SH2-containing protein tyrosine phosphatase 2
TEMED	N,N,N,-N'-Tetramethylethylene-Diamine
TGF-α	Transforming Growth Factor-alpha
TGF-β	Transforming Growth Factor-beta
Tie	Tyrosine Kinase with Immunoglobulin-like and Epidermal growth factor homology domains
TIMPs	Tissue Inhibitors of Metalloproteinases
TKrC	Tyrosine Kinase C
TNF-α	Tumour Necrosis Factor-alpha
TRL	Trilostane
TSP-1	Thromobospondin-1
TSR	Template Suppression Buffer
uPA	Urokinase Plasminogen Activator
UTR	Untranslated Region
VEGF	Vascular Endothelial Growth Factor
VEGF-R	Vascular Endothelial Growth Factor Receptor
VPF	Vascular Permeability Factor

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1. Introduction

1.1 General review of angiogenesis

1.1.1 Angiogenesis in development and growth

Angiogenesis refers to the generation of new blood vessels from preexisting vessels by remodeling of the primary plexus, thus leading to subsequent neovascularization. Angiogenesis plays a crucial role in various physiological processes, including embryonic, fetal and post-natal development and growth. However, it rarely occurs physiologically in adulthood except under strictly controlled conditions and the continuous remodeling of the adult female reproductive function. Regarding the control of female reproductive function, angiogenesis is mainly responsible for follicular and normal placental development, corpus luteum formation and repair of endometrium during the menstrual cycle. Normal tissue growth, such as in embryonic development, tissue repair processes, and the menstrual cycle, is dependent on new vessel formation for the supply of oxygen and nutrients as well as removal of waste products [Kämpfer *et al.*, 2001; see reviews by Findlay, 1986; Norrby, 1997; Griffioen & Molema, 2000].

Apart from angiogenesis, vasculogenesis can be considered to be an important determinant for early stage of vascular development and this process involves the establishment of primitive vascular network and differentiation of new endothelial cells from stem cells during embryogenesis from multipotential mesenchymal progenitors. In this process, vascular endothelial cell precursors (angioblasts) undergo differentiation from mesoderm-derived precursors (hemangioblasts), expand, and then coalesce to form a network of primitive tubules [Risau, 1997; Kämpfer *et al.*, 2001; see reviews by Ferrara, 1999; Geva & Jaffe, 2000]. Vasculogenesis results in the formation of primary capillary plexus by

endothelial cells, which are the only components of this initial lattice. This primary capillary plexus is composed of rather homogenously sized interconnected vessels and is then remodeled by a process of angiogenesis [see review by Gale & Yancopoulos, 1999]. Therefore, vasculogenesis constitutes the early stage of vascular development whereas angiogenesis forms the basis for the later stage of vascular development [Suri *et al.*, 1998].

Angiogenesis is of particular significance not only in the normal growth of both embryonic and postnatal tissues and continuous remodeling of the adult female reproductive system, but also in a variety of pathological situations such as wound healing, diabetic retinopathy, age-related macular degeneration, rheumatoid arthritis, psoriasis, cardiovascular diseases, ischemic diseases, chronic inflammation, tumour growth and metastasis. In contrast to angiogenesis, vasculogenesis is only restricted to early embryogenesis [Ferrara, 1995; Folkman, 1995; Hanahan & Folkman, 1996; Patan *et al.*, 1996a, 1996b; see review by Ferrara, 1999].

1.1.2 The process of angiogenesis

Angiogenesis mainly involves the sprouting, branching, and differential growth of blood vessels to form the more mature vascular patterns seen in the adult organism. Activation of endothelial cells, which line the vessel wall, is the first process to take place either under physiological or pathological conditions, regardless of the types of angiogenesis [Mandriota *et al.*, 1998; see reviews by Gale & Yancopoulos, 1999; Griffioen & Molema, 2000; Gerwins, Skoldenberg & Claesson-Welsh, 2000]. The development of a new capillary network requires a remarkable degree of interaction between different growth factors and occurs as an

orderly series of events. The angiogenic process begins with the disappearance of the resting state of endothelial cells and a local proteolytic degradation of the basement membrane of the vessel on the side closest to the angiogenic stimulus, followed by chemotactic migration of endothelial cells towards the site of angiogenic stimulus together with endothelial cell proliferation. Then, lumen formation begins, either by curvature of the endothelial cell or vacuole formation in the cell, followed by deposition of a new basement membrane and recruitment of perivascular cells such as smooth muscle cells and pericytes. Finally, newly formed blood vessel tubes are apparently stabilized through the migration of pericytes that provide structural support, paracrine signalling and intercellular basement membrane matrix production. Subsequently, previously avascular tissues and organs undergo vascularization and the initially homogeneous capillary plexus undergoes remodeling to form a new microcirculation. Much attention has been paid to angiogenesis-activating factors that significantly elicit an angiogenic response [Risau, 1997; Enholm *et al.*, 1997; Gerwins *et al.*, 2000; see reviews by Findlay, 1996; Geva & Jaffe, 2000].

1.1.3 Types of factors controlling angiogenesis

The mechanisms regulating vasculogenesis and angiogenesis remain to be elucidated. However, several protein factors crucial for regulation of angiogenesis have recently been identified, including growth factors, pro-angiogenic cytokines, angiogenesis modulators and inhibitors of neovascularization [see review by Norrby, 1997]. In general, angiogenesis is thought to be regulated through a balance between endogenous positive and negative signals for endothelial cell proliferation, migration, differentiation and survival [Joukov *et al.*, 1996;

Maisonpierre *et al.*, 1997]. Since the discovery of the first angiogenesis inhibitor in 1975 and the purification of the first angiogenic factor (basic fibroblast growth factor, bFGF) in 1984, at least 21 angiogenic growth factors and at least 27 angiogenesis inhibitors have been found to regulate angiogenesis (Table 1.1). As reviewed by Norrby (1997), angiogenesis inhibitors are those that could inhibit angiogenesis *in vivo* and influence the ability of a cell to produce, interact with, or degrade its surrounding matrix. These angiogenesis inhibitors may be differentially or combinatorially involved in vascular maturation [Risau, 1997].

Table 1.1 Angiogenic growth factors and angiogenesis inhibitors involved in regulation of angiogenesis*

Angiogenic growth factors	Angiogenesis inhibitors
Angiogenin	Angiostatin (plasminogen fragment)
Angiopoietin-1	Antiangiogenic antithrombin III
Del-1	Cartilage-derived inhibitor (CDI)
Epidermal growth factor (EGF)	CD59 complement fragment
Fibroblast growth factors: acidic (aFGF) and basic (bGF)	Endostatin (collagen XVIII fragment)
Follistatin	Fibronectin fragment
Granulocyte colony-stimulating factor (G-CSF)	Gro-beta
Hepatocyte growth factor (HGF) / scatter factor (SF)	Heparinases
Interleukin-3 (IL-3)	Heparin hexasaccharide fragment
Interleukin-8 (IL-8)	Human chorionic gonadotropin (hCG)
Leptin	Interferon alpha/beta/gamma
Midkine	Interferon inducible protein (IP-10)
Placental growth factor	Interleukin-12
Platelet-derived endothelial cell growth factor (PD-ECGF)	Kringle 5 (plasminogen fragment)
Platelet-derived growth factor-BB (PDGF-BB)	Metalloproteinase inhibitors (TIMPs)
Pleiotrophin (PTN)	2-Methoxyestradiol
Proliferin	Placental ribonuclease inhibitor
Transforming growth factor-alpha (TGF-alpha)	Platelet factor-4 (PF4)
Transforming growth factor-beta (TGF-beta)	Prolactin 16kD fragment
Tumor necrosis factor-alpha (TNF-alpha)	Proliferin-related protein (PRP)
Vascular endothelial growth factor (VEGF) / vascular permeability factor (VPF)	Retinoids
	Tetrahydrocortisol-S
	Thrombospondin-1 (TSP-1)
	Transforming growth factor-beta (TGF-b)
	Vasculostatin
	Vasostatin (calreticulin fragment)

*In part modified from Klagsbrun (1992); an internet website: www.angio.org/understanding/understanding.html

Although angiogenesis is driven by numerous mediators produced by different cell types under a variety of conditions, only two growth factors, vascular endothelial growth factor (VEGF) and placental growth factor (PGF), are characterised by their direct influence on endothelial cell behaviour both *in vitro* and *in vivo*. In contrast to direct angiogenic factors, indirect angiogenic factors exert their selective actions on microvascular endothelial cells *in vivo* and they are incapable of inducing capillary formation *in vitro* [see reviews by Folkman *et al.*, 1980; Norrby, 1997]. Among proangiogenic factors, only VEGF is currently held to be the major endothelial-cell-specific angiogenesis and permeability factor both in physiological and pathological conditions [Kukk *et al.*, 1996; Maisonpierre *et al.*, 1997]. In addition, VEGF differs from other endothelial growth factors (such as fibroblast growth factor and platelet-derived growth factor) in that it possesses signal sequence for secretion and is mitogenic only to endothelial cells. In this respect, VEGF is one of the best studied and the most potent proangiogenic factor. It was firstly discovered in the early eighties by Dvorak and colleagues based on its ability to induce proliferation and permeability increase of vascular endothelium [Senger *et al.*, 1983].

1.2 Roles of VEGF and its receptors in the regulation of angiogenesis

1.2.1 VEGF

The molecular aspects of signaling cascades critical for endothelial cell proliferation and migration are beginning to be delineated but much less is known about signaling cascades leading to endothelial cell differentiation. The central role of VEGF in the induction of angiogenesis in many physiological processes is now

well established [Goede *et al.*, 1998].

VEGF is a secreted homodimeric glycoprotein with molecular weight ranging from 43-46 kDa (kiloDalton) approximately. Although VEGF is encoded by a single gene located on chromosome 6, it is now well established that alternative exon splicing generates several isoforms, having, respectively, 121, 145, 165, 189 and 206 amino acid residues. These isoforms have different heparin and heparan-sulphate binding properties but with similar specificity of biological effect on endothelial cells [Enholm *et al.*, 1997; Ergun *et al.*, 1997; see reviews by Ferrara, 1996; Ferrara *et al.*, 1997; Neeman *et al.*, 1997; Norrby, 1997; Neufeld *et al.*, 1999; Ferrara, 1999; Griffioen & Molema, 2000]. Among these different VEGF isoforms, $VEGF_{165}$ is the most abundant and best-characterized isoform produced by a variety of normal and transformed cells. Transcripts encoding $VEGF_{121}$ and $VEGF_{189}$ are also detectable in normal cells and tissues expressing the VEGF gene. However, $VEGF_{206}$ is a very rare form and $VEGF_{145}$ expression is restricted to cells derived from reproductive organs. Both $VEGF_{165}$ and $VEGF_{121}$ are secreted soluble glycoproteins even though a significant fraction of $VEGF_{165}$ remains bound to the cell surface and the extracellular matrix. The unique characteristics of $VEGF_{121}$ is its weak binding affinity to heparin or extracellular matrix which is due to the lack of the amino acids encoded by exons 6 and 7 of the VEGF gene. Conversely, the longer isoforms - $VEGF_{189}$ and $VEGF_{206}$, are more basic, bind to heparin with greater affinity than $VEGF_{165}$ and are almost completely sequestered in the extracellular matrix [see reviews by Ferrara *et al.*, 1997; Neufeld *et al.*, 1999]. All of these VEGF isoforms (also known as VEGF-A), together with VEGF-B, VEGF-C, and VEGF-D, constitute the VEGF / platelet-derived growth factor (PDGF) superfamily. The ability of VEGF to induce vasodilation via

endothelial nitric oxide production and vascular permeability increase are additional features of VEGF that give its crucial role in angiogenesis initiation [Ziche *et al.*, 1997; Gerwins *et al.*, 2000]. Through these activities, VEGF allows plasma proteins to enter the tissue to form a fibrin-rich provisional network [Dvorak, 1986].

1.2.2 VEGF receptors

VEGF specifically activates the angiogenic cascade by interaction with two different forms of VEGF-specific tyrosine kinase receptors VEGFR-1/Flt-1 (fms-like tyrosine kinase-1) and VEGFR-2/KDR (kinase insert domain-containing receptor). The latter corresponds to Flk-1 (fetal liver kinase-1) in the mouse and TKrC (tyrosine kinase C) in the rat [Joukov *et al.*, 1996; Ergun *et al.*, 1997; Korpelainen *et al.*, 1998; Goede *et al.*, 1998; Papapetropoulos *et al.*, 1999; Bi, Drake & Schwarz, 1999; see reviews by Ferrara *et al.*, 1997; Norrby, 1997; Yancopoulos *et al.*, 2000; Griffioen & Molema, 2000]. VEGFR-1 is a 180-kDa transmembrane glycoprotein and alternative splicing of this receptor generates a shorter soluble protein containing only six first extracellular immunoglobulin homology domains followed by 31 unique amino acid residues. Interestingly, there are two functional VEGFR-2 forms expressed in retinal, as a result of alternative splicing and the molecular weight of VEGFR-2 is about 230 kDa in general [Petrova, Makinen & Alitalo, 1999; see review by Neufeld *et al.*, 1999]. Consistent with the specificity of action of this growth factor family, these receptors are expressed predominantly in vascular endothelium, and to a lesser extent on monocytes / macrophages and certain tumor cell types. It was observed that the migration of monocytes in response to VEGF was mediated by VEGFR-1 [Suri *et*

al., 1998; Korpelainen *et al.*, 1998; see reviews by Ferrara *et al.*, 1997; Ferrara, 1999; Neufeld *et al.*, 1999; Yancopoulos *et al.*, 2000; Griffioen & Molema, 2000].

Besides VEGFR-1 and VEGFR-2, the receptor for VEGF-C and VEGF-D has been described: VEGFR-3 (Flt-4) exists as 4.5- and 5.8-kb transcripts resulting from alternative 3' polyadenylation signals [Joukov *et al.*, 1996; Petrova, Makinen & Alitalo, 1999; see reviews by Ferrara *et al.*, 1997; Yancopoulos *et al.*, 2000; Griffioen & Molema, 2000]. The expression of the longer form is detectable in the majority of cells and tissues and this longer form is capable of encoding 65 additional amino acid residues. Compared with VEGFR-1 and VEGFR-2, VEGFR-3 is mainly expressed on lymphatic endothelium, for which it seems to be critical in mediating VEGF action in stimulating proliferation of lymphatic endothelial cells [Kukk *et al.*, 1996; see review by Yancopoulos *et al.*, 2000].

All of these VEGF receptors constitute type III transmembrane receptor tyrosine kinases of the platelet-derived growth factor (PDGF) receptor subfamily, and they undergo ligand-induced dimerization to allow the activation of complex intracellular signaling pathways [Petrova, Markinen & Alitalo, 1999]. The interaction of VEGF-A with VEGFR-2 has been extensively investigated, since the angiogenic effect of VEGF in causing porcine endothelial cell proliferation as well as chemotactic migration and sprouting activity seems to be mediated largely by the VEGFR-2 but not VEGFR-1. The latter appears to play a more important role in endothelial organization during development [Joukov *et al.*, 1996; see reviews by Korpelainen *et al.*, 1998; Yancopoulos *et al.*, 2000; Griffioen & Molema, 2000].

VEGFR-2 is the most abundant in proliferating endothelial cells of vascular sprouts and branching vessels of embryonic tissues, and to a lesser extent in adult tissues, but it can also be activated in response to VEGF-C. VEGF-C induced

autophosphorylation of VEGFR-2 was evident in the investigation of the activity of recombinant VEGF-C *in vitro* by stimulation of VEGFR-3- and VEGFR-2-expressing cells, followed by receptor autophosphorylation analysis. The possible effects of VEGF-C interaction with VEGFR-2 would be to cause striking changes in the morphology, actin reorganization and membrane ruffling of porcine aortic endothelial (PAE) cells overexpressing VEGFR-2 [Joukov *et al.*, 1996; Kukk *et al.*, 1996].

1.2.3 Regulation of VEGF expression by hypoxia and nitric oxide

Hypoxia has been proposed to play an important role in the regulation of VEGF gene expression, both *in vitro* and *in vivo* [see reviews by Ferrara *et al.*, 1997; Ferrara, 1999; Neufeld *et al.*, 1999]. The upregulation of VEGF expression by hypoxia is attributable to the induction of VEGF gene transcription by the binding of hypoxia-inducible factor-1 (HIF-1) to an HIF-1 binding site located in the VEGF promoter. The influence of hypoxia on VEGF gene expression has been investigated in numbers of studies and identified the increased VEGF mRNA stability as a significant post-transcriptional component in addition to transcriptional activation, probably via the binding of proteins to sequences located in the 3' untranslated region (UTR) of the VEGF mRNA. The stimulatory effect of hypoxia on VEGF gene transcription can also occur as a result of the regulation of the translation of uncapped mRNA initiated from an active internal ribosomal entry site (IRES) located downstream of an alternative initiation site in the 5' UTR of the VEGF mRNA. These phenomena strongly point to an early involvement of VEGF in the angiogenic response.

Neufeld *et al.* (1999) has recently reviewed that hypoxia-induced VEGF

production is responsible for angiogenesis initiation, using the development of the retina and the associated network of retinal blood vessels as a typical example. Similar mechanisms of vascular development also operate in other organs. The astrocytes respond to hypoxia by producing VEGF, which then initiates an angiogenic response during the development of the retina. The resulting VEGF concentration gradient stimulates the growth and continuous outward migration of new blood vessels toward the VEGF-producing astrocytes. There is a decreased VEGF production to a certain threshold concentration by the astrocytes that are the sensors of hypoxia upon the arrival of the blood vessels to prevent apoptosis of the endothelial cells and stabilize the newly formed blood vessels. Inductions of smooth muscle cell/pericyte loss render the remaining blood vessels insensitive to the absence of VEGF.

VEGF partly responds to nitric oxide and cGMP-mediated activation of the mitogen-activated protein kinase (MAPK) family by influencing endothelial cell proliferation [Risau, 1997; see reviews by Ferrara *et al.*, 1997; Neufeld *et al.*, 1999; Griffioen & Molema, 2000]. VEGF has a dual function involving both angiogenesis initiation and endothelial cell proliferation. Nitric oxide is known to increase vascular permeability and induce vasodilation by mediating the effects of VEGF. These influences result from a positive feedback loop between VEGF and nitric oxide. In support of the view of the growth-promoting effect of VEGF on vascular endothelial cells, it has been shown that nitric oxide synthase (NOS) inhibitors inhibit the mitogenic effect of VEGF on human umbilical vein endothelial cells (HUVEC) [Papapetropoulos *et al.*, 1997] and bovine endothelial cells isolated from coronary postcapillary venules [Morbidelli *et al.*, 1996].

The underlying mechanism for MAPK pathway to induce endothelial cell

proliferation and migration appears to be due to the activation of NOS upon VEGF stimulation, followed by a subsequent increase in cGMP. After the increase in nitric oxide level upon VEGF stimulation, there is an increase in intracellular calcium levels and an activation of serine kinase Akt/PKB so as to activate endothelial NOS (eNOS) and phosphorylate eNOS in a calcium-dependent manner respectively (mentioned later in Section 1.2.5). Some adaptor proteins such as Grb2, Shc, and Nck are thought to be involved in the VEGF-promoted activation of MAPK cascade in porcine aortic endothelial cells expressing recombinant VEGFR-2 [see reviews by Petrova, Makinen & Alitalo, 1999; Neufeld *et al.*, 1999]. VEGF-mediated signaling is subsequently able to confer a proliferative signal in endothelium through a physical association of the SHP-1 and SHP-2 SH2 protein-tyrosine phosphatases with VEGFR-2. This induces endothelial expression of extracellular matrix (ECM)-degrading enzymes required for the breakdown of the basement membrane of blood vessels. Loss of integrity of the basement membrane is critical for the very initial phases of angiogenesis, the initiation of cell proliferation and cell migration.

1.2.4 Signal transduction mechanisms of VEGFR-1 and VEGFR-2

Current research interest on angiogenesis control involves the elucidation of different signal transduction properties of VEGFR-1 and VEGFR-2, and the regulation of their expression. Of great importance is the recent findings documented by Ferrara (1999), Petrova, Makinen & Alitalo (1999) and Yancopoulos *et al.* (2000) that VEGFR-1 might act as a ligand-binding molecule or a decoy receptor rather than a signal-transducing receptor during angiogenesis or might suppress signaling through VEGFR-2. This notion is supported by numbers of previous studies, which demonstrated a lack of direct mitogenic signal in transfected

NIH 3T3 cells expressing VEGFR-1 upon VEGF stimulation as well as the absence of permeability-enhancing properties of VEGFR-1-transfected endothelial cells. These can be explained by weak tyrosine phosphorylation of VEGFR-1 and a high affinity of placental growth factor (PlGF) to VEGFR-1 but not to VEGFR-2 respectively. Park *et al.* (1999) have demonstrated a negative regulation of VEGF activity on endothelial cells by sequestering and rendering VEGF less available to VEGFR-2. In spite of the relatively higher affinity of VEGF for VEGFR-1 than for VEGFR-2, research has focused on VEGF-induced signal transduction in endothelial cells by both receptors.

Studies involving targeted disruption of VEGF, VEGFR1 or VEGFR2 genes in mice have provided the best evidence for the critical role of VEGF and its receptors in the development of the embryonic vasculature. Nevertheless since these mutant mice exhibited different developmental defects in their vascular system and died at different ages, the data suggested to us that differences exist for the respective role of VEGF and its two receptors in endothelial cell differentiation and proliferation. Mice lacking even a single VEGF allele exhibit embryonic lethality due to severe vascular abnormalities, implicating a simple half-dosage effect on embryonic lethality [Suri *et al.*, 1996; see reviews by Ferrara, 1999; Petrova, Makinen & Alitalo, 1999; Neufeld *et al.*, 1999; Yancopoulos *et al.*, 2000]. VEGFR-2 knockout mice provide the most elegant demonstration of VEGF as a signal in embryonic angiogenesis. Hematoangioblast progenitors are severely disrupted, followed by subsequent embryonic lethality between day 8.5 and 9.5, because of failure in both endothelial cell differentiation and vascular development. Although embryonic lethality around day 8.5 has also been observed in VEGFR-1 knockout mice, VEGFR-1 null phenotype is distinct from that of the VEGFR-2 knockouts

[Carmeliet *et al.*, 1996; Ferrara *et al.*, 1996]. Consistent with the critical role of VEGFR-1 in down-regulating VEGF activity, excess levels in endothelial cells and normal hematopoietic progenitors seemingly develop in VEGFR-1 knockout mice but they abnormally coalesce into disorganized tubules following migration and proliferation. As reviewed by Gale & Yancopoulos (1999) and Yancopoulos *et al.* (2000), VEGFR-1 as a decoy receptor was further confirmed by normal vascular development in mice engineered to express only a truncated form of VEGFR-1 lacking its tyrosine kinase domain.

1.2.5 Anti-apoptotic effect of VEGF on endothelial cells as a result of signal transduction of VEGFR-2

The protection of endothelial cells against serum deprivation and tumor necrosis factor α-induced apoptosis is dependent upon the activation of VEGFR-2. The activation of phosphatidylinositol 3'-kinase (PI 3'-kinase) can contribute significantly to the signal transduction of such VEGF-induced prosurvival effect in endothelial cells, probably via activation of its downstream target serine kinase Akt/PKB [see reviews by Ferrara, 1999; Petrova, Makinen & Alitalo, 1999]. However, the activation of PI 3'-kinase does not seem to induce endothelial cell proliferation efficiently. This argument is evident from the studies about the requirement of VEGFR-2 activation for the antiapoptotic effects of VEGF for HUVEC in serum-free conditions. In addition, the activation of antiapoptotic serine-threonine kinase (Akt, also known as protein kinase B) was observed in HUVEC only upon stimulation with a VEGFR-2-selective VEGF mutant rather than with PlGF or a VEGFR-1-selective mutant, demonstrating the crucial role of VEGFR-2 in mediating VEGF-induced endothelial cell survival. Akt/PKB pathway can be

activated in endothelial cells by the increase in intracellular calcium levels, because of VEGF-mediated nitric oxide production (as explained earlier in Section 1.2.3). Apart from PI 3'-kinase, recent studies have provided evidence in support of the hypothesis for the involvement of VEGFR-2 in a multimeric complex together with the adherens junction protein VE-cadherin and β-catenin in regulation of endothelial cell apoptosis.

1.3 Angiopoietins

Despite a pivotal role of VEGF in the earliest stages of vasculogenesis and subsequent angiogenesis, it seems to work in complementary and coordinated fashion during vascular development with another family of growth factors specific for the vascular endothelium – angiopoietin [Suri *et al.*, 1998; Valenzuela *et al.*, 1999; see reviews by Gale & Yancopoulos, 1999; Griffioen & Molema, 2000; Yancopoulos *et al.*, 2000]. The Tie (*t*yrosine *k*inase containing *i*mmunoglobulin-like loops and *e*pidermal growth factor similar domains) receptors, Tie 1 and Tie 2, constitute a second family of endothelial cell-specific receptor tyrosine kinases (RTKs) that are specifically expressed in developing vascular endothelial cells. Although a functional ligand for the Tie 1 receptor have not yet been identified, the Tie 1 receptor is known to be closely related to the Tie 2 receptor. Angiopoietin 1 (Ang-1) and Angiopoietin 2 (Ang-2) and, more recently, Angiopoietin 3 (Ang-3) and Angiopoietin 4 (Ang-4) have been discovered as ligands for receptor tyrosine kinase Tie 2. Compared to VEGF, angiopoietins seem to act primarily in the later stages of vessel growth and remodeling. Ang-1 induces Tie 2 phosphorylation in cultured endothelial cells, whereas Ang-2 does not phosphorylate Tie 2. Alternatively, Ang-2 inhibits the Ang- 1-induced Tie 2 phosphorylation in vascular endothelial cells. In

other words, Ang-2 appears to be a naturally occurring antagonist for Ang-1 and its Tie 2 receptor. Mouse Ang-3 and human Ang-4 belong to a novel subfamily of angiopoietins and represent the mouse and human counterparts of the same gene locus, in which Ang-3 acts as an antagonist and Ang-4 as an agonist of receptor tyrosine kinase signaling. Their activities have not been fully explored.

1.3.1 Angiopoietin 1 (Ang-1)

Long after the discovery of VEGF, a recent explosion of newly discovered growth factors, angiopoietins, specific for the vascular endothelium is especially important in understanding the mechanisms that govern the later stages of angiogenesis. The normal role of Angiopoietin 1 (Ang-1) has been investigated by gene deletion and dominant negative transgenic experiments. Mouse embryos null for Ang-1 or a functional angiopoietin receptor, Tie 2, display a picture quite different from knockout embryos lacking VEGF or VEGFR-2 expression, with development of a rather normal primary vasculature but failure of normal endothelial cell adherence and interaction with underlying supporting cells and extracellular matrix [Sato *et al.*, 1995; Suri *et al.*, 1996]. Then, normal remodeling events of this primitive vasculature are severely perturbed, probably leading to subsequent regression. Also, similar heart defects were observed in both Ang-1- and Tie 2-null mouse embryos, with detachment of the endocardium from the underlying myocardium, failure of trabeculae formation as well as vascular remodelling. Therefore, targeted inactivation of the Ang-1 gene revealed its obligatory role in proper interactions between endothelial cells and supporting cells, rather than as an instructive signal of vascular remodeling, thereby rendering them responsive toward other critical signals from their environment.

As reviewed by Geva & Jaffe (2000), the human Ang-1 gene has been assigned to chromosome 8 and encodes a unique 498-amino acid glycoprotein. Ang-1 is known to be an endogenously secreted glycoprotein from the pericytes and vascular smooth cells around endothelial cells. Based on preliminary biochemical analyses, Ang-1 was shown to consist of a coiled-coil domain in the amino terminus and a fibrinogen-like domain at the carboxy terminus. In transfection studies, Ang-1 recombinant protein isolated from COS-7 culture supernatant gives a molecular weight in the range of 58-75 kDa (kiloDalton). The amino acid sequence of Ang-1 appears to contain several potential glycosylation sites, suggesting that the higher molecular size product (~ 75 kDa) could represent the glycosylated form of Ang-1 [Hanahan, 1997; Kwak *et al.*, 1999; see review by Griffioen & Molema, 2000].

Intriguingly, unlike VEGF, Ang-1 does not stimulate endothelial cell proliferation nor mediate tubule formation but it likely plays a later role in endothelial cell maturation in synergy with VEGF [Mandriota & Pepper, 1998; Suri *et al.*, 1998; Huang *et al.*, 2000; Papapetropoulos *et al*, 2000; Kim *et al.*, 2000b; Kwak *et al.*, 2000; see reviews by Gale & Yancopoulos; 1999; Geva & Jaffe, 2000; Yancopoulos *et al.*, 2000]. Nevertheless, *in vitro* studies have revealed that Ang-1 potently elicits sprouting and chemotactic response of endothelial cells throughout the vascular system as well as network formation [Witzenbichler *et al.*, 1998; Kwak *et al.*, 1999]. Consistent with the action of Ang-1 in sprouting and branching *in vitro* are recent observations, transgenic overexpression of Ang-1 results in pronounced hypervascularization, probably via the inhibition of normal vascular pruning and stimulation of vascular remodelling events [Koblizek *et al.*, 1998; Suri *et al.*, 1998; see reviews by Gale & Yancopoulos, 1999; Yancopoulos *et al.*, 2000; Geva & Jaffe, 2000]. The mechanisms for Ang-1 to inhibit endothelial cell apoptosis include the

recruitment of phosphatidylinositol 3'-kinase (PI 3'-kinase), downstream activation of the Akt signal transduction pathway, and/or upregulation of a broad spectrum apoptosis inhibitor, survivin [Suri *et al.*, 1998; Kwak *et al.*, 1999; Kim *et al.*, 1999a; Papapetropoulos *et al.*, 1999; Thurston *et al.*, 2000; Kim *et al.*, 2000b; Papapetropoulos *et al.*, 2000; see reviews by Gale & Yancopoulos, 1999; Yancopoulos *et al.*, 2000].

The recent discovery of four different isoforms of Ang-1 (1.5, 1.3, 0.9, and 0.7 kb) has provided greater complexity to the molecular and cellular mechanisms of angiogenesis. It has now been well established that alternative exon splicing of the full-length Ang-1 gene forms the basis for this molecular heterogeneity. The presence of a hydrophobic leader peptide offers the potential for all four isoforms to be secreted from cells. Ang-0.7 kb isoform is a truncated form derived from two alternatively spliced regions of the full-length Ang-1 but it lacks parts of both the coiled-coil and the fibrinogen-like domains. Like Ang-0.7 kb isoform, Ang-0.9 kb isoform lacks most of the coiled-coil domain. Interestingly, there is a coiled-coil multermerization domain in Ang-1.3 kb isoform even though it lacks its purported receptor-binding domain [Huang *et al.*, 2000].

The actions of Ang-1 have been found to signal through the endothelial cell-specific Tie 2 receptor tyrosine kinase, probably via its fibrinogen-like domain [Maisonpierre *et al.*, 1997; Hanahan, 1997; Kwak *et al.*, 1999; Procopio *et al.*, 1999; Kim *et al.*, 2000b; Kwak *et al.*, 2000; see reviews by Griffioen & Molema, 2000; Yancopoulos *et al.*, 2000]. In addition to the full-length Ang-1 (Ang-1.5 kb), three alternatively spliced species of Ang-1 mRNA have been discovered (Ang-1.3 kb, Ang-0.9 kb, and Ang-0.7 kb) in the megakaryocyte cell line CHRF. Ang-1.5 kb isoform is the primary activating ligand for this Tie 2 receptor. In agreement to the

structure of Ang-1.3 kb isoform, this isoform was found to interact with Ang-1.5 kb isoform via its coiled-coil multimerization domain. Similarly, Ang-0.9 kb isoform interferes with the binding of Ang-1.5 kb isoform to Tie 2 receptor, suggesting that both Ang-1.3 kb isoform and Ang-0.9 kb isoform probably act as dominant negative isoforms for the full-length Ang-1.5 kb isoform. A relatively unexplored area of study for these isoforms involves their mechanism of action these isoforms *in vivo* and how Ang-1.3 kb and Ang-0.9 kb isoforms can interfere with the binding of Ang-1.5 kb isoform to Tie 2. Also, it is still unclear as to whether any of the isoforms interact (either activate or inhibit) as heteromultimers with related angiogenic species [Huang *et al.*, 2000].

1.3.2 Angiopoietin 2 (Ang-2)

Shortly after the discovery of Angiopoietin 1, another factor closely related to it, termed Angiopoietin 2 (Ang-2), was cloned from low-stringency screening of human and mouse cDNA libraries, using mouse Ang-1 cDNA as a probe. Transgenic studies have generated direct evidence for the importance of Ang-2 for embryonic vasculogenesis and angiogenesis. Similar to Tie 2- or Ang-1-null mice, transgenic mice overexpressing Ang-2 during embryogenesis exhibit embryonic lethality as a result of severe disruption of vessel structure and peri-endothelial support cells. These observations have clearly established a significant role of Ang-2 as a natural antagonist for the Ang-1/Tie 2 interaction [Maisonpierre *et al.*, 1997; Hanahan, 1997]. Other evidence in support of this argument came from the observations involving the inhibition of Ang-1 activity in endothelial cells via a fourfold to eightfold molar excess of Ang-2 as well as the blockage of Ang-1 chemotactic activity for endothelial cells by Ang-2 in a dose-dependent manner

[Maisonpierre *et al.*, 1997; Witzenbichler *et al.*, 1998].

Structurally, the amino acid sequences of Ang-2 and Ang-1 are about 60% identical [Maisonpierre *et al.*, 1997; Stratmann *et al.*, 1998; Witzenbichler *et al.*, 1998]. Most notably, both Ang-1 and Ang-2 bind with similar affinity to Tie 2 receptor tyrosine kinase. However, in the study of cultured endothelial cells, receptor phosphorylation of Tie 2 appears to be mediated through Ang-1 but not Ang-2. The human Angiopoietin 2 gene has been assigned to chromosome 8 [Cheung *et al.*, 1998]. In general, Ang-2 shares a striking structural similarity with Ang-1; that is, both of them have a characteristic protein structure with an NH_2-terminal coiled-coil domain and a COOH-terminal fibrinogen-like domain together with a secretion signal peptide. However, unlike the Ang-1 protein, the inferred Ang-2 protein is about 496 amino residues long [Maisonpierre *et al.*, 1997; Geva & Jaffe, 2000]. Immunoprecipitation analysis of *de novo* Ang-2 protein synthesis in ^{35}S-labeled cell lysates revealed a single band of 55 kDa approximately. However, the calculated molecular weights of the non-glycosylated and glycosylated forms of Ang-2 protein have been reported to be 57 kDa and 70 kDa respectively, since Ang-2 contained several potential glycosylation sites [Oh *et al.*, 1999].

The prominent expression of Ang-2 at sites of vascular remodeling as well as at the leading edge of invading vascular sprouts strongly implies that Ang-2 plays a facilitative role in vascular destablization, sprouting and angiogenic remodeling [Hanahan, 1997; Maisonpierre *et al.*, 1997; Asahara *et al.*, 1998; Mandriota & Pepper, 1998; Oh *et al.*, 1999; see reviews by Gale & Yancopoulos, 1999; Yancopoulos, 2000]. In fact, autocrine induction of Ang-2 in endothelium inhibits constitutive stabilizing action or the maturation function of paracrine Ang-1, thereby reverting vessels to a more plastic and tenuous state. Such inhibition leads to

cessation of vessel growth and even causes frank vascular regression in the absence of other associated growth factors. Conversely, destabilization by Ang-2 in the presence of high VEGF levels is associated with cessation of vascular regression, and onset of continued remodeling or the initiation of vascular sprouting.

In addition to normal physiological role of Ang-2 in angiogenesis, Ang-2 participates in gastric cancer angiogenesis, probably via corporation with VEGF [Etoh *et al.*, 2001]. This is evident from the intense autocrine expression of Ang-2 in ECs of tumour-associated vessels. Both autocrine and paracrine Ang/Tie 2 pathways may be responsible for the regulation of the induction of proteases in ECs by Ang-2 during tumour angiogenesis under the presence of VEGF. These signaling pathways are very similar to the model for the coordinated interaction between the VEGF and angiopoietin families mediating endothelial movement (as mentioned later in Section 1.4). The regulation of proteases such as MMP-1 (matrix metalloproteinase-1), MMP-9 (matrix metalloproteinase-9), and uPA (urokinase plasminogen activator) by common promotor regions c-Ets1 (c-erythroblast transformation specific domain 1) represents a crucial prerequisite for a tumour-associated angiogenic response in gastric carcinoma. The presence of Ang-1 is found to inhibit the activity of c-Ets1 and subsequent production of these proteases. However, Ang-2, the natural antagonist for Tie 2, is able to inhibit autophosphorylation of Tie 2 receptor by Ang-1 in human ECs. When VEGF is expressed in the presence of Ang-2, Ang-2 significantly upregulates these proteases to degrade vessel basement membranes via activation of c-Ets1 to enhance tumour progression. A dysregulated balance for Ang-2/VEGF mRNA expression is observed in cancer cells. Thus, angiogenesis in human gastric carcinoma is characterized mainly by the overexpression of Ang-2 together with VEGF expression and Ang-2 can potentiate VEGF-induced

angiogenesis, probably by enhancing the initiation of neovascularization.

Despite the antagonizing effect of Ang-2 on Ang-1-induced receptor phosphorylation in endothelial cells, Ang-2 is able to activate Tie 2 expressed in NIH 3T3 fibroblasts or hemangioblast precursor cells [Maisonpierre *et al.*, 1997; Witzenbicheler *et al.*, 1998]. Interestingly, the equivalent contribution of Ang-1 and Ang-2 to Tie 2 phosphorylation [Maisonpierre *et al.*, 1997] and a specific chemotactic response to both Ang-1 and Ang-2 [Witzenbicheler *et al.*, 1998] were previously demonstrated in an experiment performed in NIH 3T3 fibroblasts expressing transfected Tie 2 receptors. On the basis of these observations, Ang-2 acts as an antagonist for Tie 2 receptor in endothelial cells but not in other cell types.

Since hypoxia is well known to be a primary inducer of neovascularization in a variety of pathological conditions, such as tumour angiogenesis, collateral vessel formation in cardiovascular diseases, and proliferative retinal neovascularization, and it has been shown that hypoxia can trigger an up-regulation of VEGF expression (as mentioned in Section 1.2.3), it would be of interest to discuss whether hypoxia and VEGF can selectively enhance Ang-2 expression in endothelial cells. First of all, the up-regulation of Ang-2 expression by VEGF has been well demonstrated in bovine endothelial cells and mouse model of ischemia-induced retinal angiogenesis *in vivo*. The mechanism by which VEGF increases Ang-2 expression is however partially understood. Using several inhibitors of specific signal transduction pathways, Oh *et al.* (1999) revealed predominant roles of both MAPK activation and tyrosine phosphorylation in VEGF-induced Ang-2 mRNA expression of bovine retinal endothelial cells (BRECs), but a more minor contribution of the protein kinase C-dependent pathway.

In addition to VEGF, hypoxia has also been reported to selectively up-

regulate Ang-2 mRNA expression and protein synthesis of Ang-2 in BRECs [Oh *et al.*, 1999]. The exposure of BRECs to hypoxic conditions results in a peak Ang-2 mRNA expression at 2 hours after treatment, followed by a gradual decline to the basal level after 4 hours of stimulation. In view of the fact that hypoxia is the major stimulus for VEGF induction and VEGF increases Ang-2 expression in the study, it must be further determined whether hypoxia-induced VEGF is involved in the observed hypoxic regulation of Ang-2 in BRECs. Further experiment confirmed that in BRECs under normoxic or hypoxic conditions, there was no significant effect of the anti-VEGF neutralizing antibody on Ang-2 mRNA expression. It would then appear that hypoxia produced a direct stimulation of Ang-2 mRNA levels in BRECs. In agreement with the above findings, Mandriota & Pepper (1998) used RNase protection assay and demonstrated that 30 ng/mL of VEGF and hypoxia induced a 2.9-fold and a 5.6-fold increase in Ang-2 mRNA levels in adrenal cortex-derived BME cells respectively. A possible explanation for the observed upregulation of Ang-2 expression by hypoxia and VEGF in hypoxic retina and neovascular vessels *in vivo* is that since Ang-2 has been reported to be a natural antagonist for Tie 2 receptor, the angiogenic stimuli of hypoxia and VEGF may probably well deteriorate the integrity of the vasculature by suppressing Ang-1 activation of Tie 2 receptor.

Human umbilical vein endothelial cell (HUVEC) cDNA analysis has yielded the identification of a novel alternatively splice variant of the human Ang-2 gene ($Ang2_{443}$) [Kim *et al.*, 2000a]. It is derived from alternative splicing in which part of the coiled-coil domain (amino acids 96-148) is deleted and hence it lacks 53 amino acids. The structure of Ang-2_{443} contains a secretory signal peptide and four of the six potential glycosylation sites. Ang-2_{443} binds to the Tie 2 receptor but it is incapable of stimulating Tie 2 phosphorylation. It may therefore act as a functional

antagonist of Ang-1-induced Tie 2 phosphorylation by interfering with binding of Ang-1 to Tie 2 receptor.

The functional roles and expression pattern of Ang-2_{443} have also been described. It is of interest that Ang-2_{443} mRNA was mainly present in primary endothelial cells, several nonendothelial tumor cell lines, and primary tumor tissues. Macrophage differentiation might be probably associated with temporary upregulation of Ang-2_{443} expression. Such distribution pattern is consistent with a functional role for Ang-2_{443} as a major regulator of angiogenesis during some tumorigenic and inflammatory processes.

Three different Ang-2 mRNA species encoding Ang-2A, Ang-2B and Ang-2C isoforms are produced by alternative splicing of the chicken Ang-2 gene [Mezquita *et al.*, 1999, 2000]. Although Ang-2 isoforms have been successfully identified in two different species, human and chicken, it remains to be elucidated whether Ang-2 isoforms can be expressed in other species such as rat and mouse.

1.3.3 Angiopoietins 3 and 4 (Ang-3 and Ang-4)

Using homology-based cloning approaches, the angiopoietin family has recently been expanded following the isolation of two new members, mouse-specific Angiopoietin 3 (Ang-3) and human-specific Angiopoietin 4 (Ang-4) [Valenzuela *et al.*, 1999; Brown *et al.*, 2000]. Similar to Ang-1 and Ang-2, both Ang-3 and Ang-4 possess characteristic protein structures containing coiled-coil domains in the N-terminal region, fibrinogen-like domain as well as a secretory signal peptide. An important message from chromosomal localization analyses of all of the angiopoietins in mouse and human is that both Ang-3 and Ang-4 represent the mouse and human counterparts of the same gene locus. However, compared with 99% and

87% amino acid identity within the fibrinogen-like domains (FDs) of mouse and human counterparts of Ang-1 and Ang-2, the structural divergence of mouse Ang-3 and human Ang-4 is evident from only 54% and 65% overall amino acid identity within their FDs respectively. On the basis of such amino acid identity, it seems likely that the similarity between mouse Ang-3 and human Ang-4 is much less than that between the mouse and human counterparts of Ang-1 and Ang-2, suggestive of diverging functions of the gene products in the two species.

Although the respective roles of both mouse Ang-3 and human Ang-4 in angiogenesis and vascular maintenance are not yet fully elucidated, it is now well appreciated that they exert opposing activities on the Tie 2 receptor [Valenzuela *et al.*, 1999]. The observation that Ang-3, like Ang-2, was able to phosphorylate Tie 2 when expressed by fibroblasts, indicates that in endothelial cells other regulatory mechanism(s) prevail leading to their antagonistic activity. On the contrary, Ang-4, as is Ang-1, acts as a Tie 2 agonist in either human or mouse endothelial cells as well as mouse fibroblasts. Based on this current evidence, both Ang-3 and Ang-4 appear to be true members of the angiopoietin family, as judged by their specific binding to the Tie 2 receptor, and it would be tempting to speculate their differences in the biological effects at the molecular and cellular level.

1.4 Interaction among VEGF, angiopoietin and Tie in maintenance of vasculature

Highly coordinated interaction between two families of angiogenic factors, vascular endothelial growth factor (VEGF) and angiopoietins, has provided novel and important insights into physiological regulation of vasculogenesis, vessel

maturation and maintenance, angiogenesis, and regression during vascular development, as schematically shown in Figure 1.1 [Hanahan, 1997; Maisonpierre *et al.*, 1997; Kwak *et al.*, 1999; Holash *et al.*, 1999; see reviews by Peters, 1998; Gale & Yancopoulos, 1999; Yancopoulos *et al.*, 2000]. Such model system still maintains the position of VEGF as the most critical molecular player of vascular development, with VEGF-mediated activation of VEGFR-2 eliciting differentiative, proliferative and chemotactic responses from endothelial cells, followed closely by the induction of endothelial cell interactions and capillary tube formation via VEGF/VEGFR-1 interaction. Most notably, Angiopoietin 1 (Ang-1)/Tie 2 system is a predominant and potent regulator of vascular function and integrity, acting to mediate vessel maturation from simple endothelial tubes into more elaborate vascular structures composed of several cell types and subsequently to maintain the quiescence and stability of the mature vasculature. The enhancement of endothelial cell interactions with surrounding support cells and matrix is a prerequisite to form a stable vasculature. Unlike Ang-1, Angiopoietin 2 (Ang-2) has been identified as a naturally occurring antagonist of Ang-1 activation of Tie 2 during angiogenesis even though Ang-2 can still promote vascular remodeling. Ang-2 is specifically required to counteract Ang-1-mediated blood vessel stability by allowing the endothelial cells to revert to a more plastic and destabilized state reminiscent of developing vessels. Such destabilized vessels could be prone to two fates. On the one hand, these vessels become more sensitive to angiogenic changes induced by simultaneously available angiogenic factors such as VEGF, essentially recapitulating an early embryonic situation in which VEGF acts prior to the involvement of Ang-1. On the other hand, the absence of VEGF co-expression triggers endothelial cell death, likely by apoptosis, followed by subsequent vessel regression.

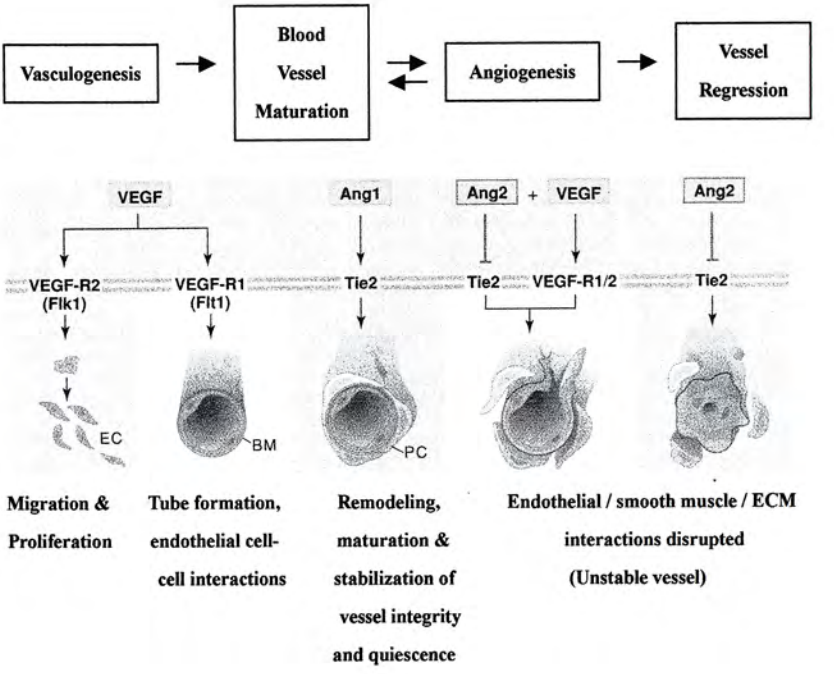


Figure 1.1 **Model for the co-ordinated and complementary roles of VEGF and the angiopoietins during vascular development and remodeling**
***In part modified from Hanahan (1997)**

1.5 Tyrosine kinase with immunoglobulin and EGF factor homology domains - Tie 1 and Tie 2

The biological actions of angiopoietins have been found to manifest through one form of transmembrane tyrosine kinase receptors (as mentioned earlier in Section 1.3), termed Tie 2. Both Tie 1 and Tie 2 receptors are absolutely critical for the later stage of angiogenesis rather than vasculogenesis and they belong to the receptor tyrosine kinase (RTK) family specifically expressed in developing vascular endothelial cells [Maisonpierre *et al.*, 1993; Sato *et al.*, 1995; Partanen *et al.*, 1996; Asahara *et al.*, 1998; Witzenbichler *et al.*, 1998; Papapetropoulos *et al.*, 1999; Uchida *et al.*, 2000]. Both Tie 1 gene and Tie 2 gene have been localized to mouse chromosome 4 homologous to human chromosome 1p [Sato *et al.*, 1993; Korhonen *et al.*, 1994]. Different from other tyrosine kinase receptors, this putative RTK is composed of two intracellular tyrosine kinase domains and two unique extracellular immunoglobulin-like domains straddling three tandem EGF-like motifs, followed by three fibronectin (FN) type III repeat sequences located near the transmembrane domain [Maisonpierre *et al.*, 1993; Iwama *et al.*, 1993; Sato *et al.*, 1993; Hashiyama *et al.*, 1996; Batard *et al.*, 1996; Geva & Jaffe, 2000]. The extracellular domains of both Tie 1 and Tie 2 share about 50% amino acid similarity in all three EGF-like repeats, but minimal homology within their two immunoglobulin-like loops (20-30%). This is in contrast to 80% amino acid identity within the cytoplasmic domains of Tie 1 and Tie 2.

The mechanism of action of Tie proteins is similar to that of other growth factor transmembrane tyrosine kinase receptors [Maisonpierre *et al.*, 1993; Sato *et al.*, 1993; Wong *et al.*, 1997; Jones *et al.*, 1999; Geva & Jaffe, 2000]. The binding of either diffusible or cell-associated ligand to EGF-like repeats, immunoglobulin-like

loops, and FN III-like repeats in the extracellular domain of the Tie 2 protein induces receptor activation, an event that immediately leads to ligand-dependent receptor autophosphorylation. As with VEGF receptor signaling, this autophosphorylation enables the p85 subunit of the lipid kinase phosphatidylinositol (PI) 3'-kinase, the SH2-containing protein tyrosine phosphatase 2 (SHP2), and the adaptor proteins - growth factor receptor-bound protein 2 (Grb2), Grb7, and Grb14, to bind probably via their Src-homology-2 (SH2) domains *in vitro*, and subsequently activate intact PI 3'-kinase signal transduction pathway. This pathway is partially implicated in promotion of endothelial cell migration and survival, suggestive of the dependence of endothelial cell maintenance upon PI 3'-kinase signaling pathway through Tie 2. Consistent with constitutive expression of Tie 2 in both angiogenic endothelium and quiescent vasculature in the adult rat, it appears that tyrosine phosphorylation of Tie 2 plays a facilitative role not only in angiogenesis but also in vascular maintenance [Wong *et al.*, 1997]. Interestingly, a multisubstrate docking site Tyr^{1100} located in the C-terminal tail of Tie 2 permits Grb2, Grb7 and p85 (via its C-terminal SH2 domain) to associate with the receptor both *in vivo* and *in vitro* after Ang-1 stimulation of Tie 2. The observation of endothelial cell migration in response to Ang-1 mediated by p85 signaling and Tyr^{1100} requirement of both p85 and Grb7 phosphorylation strengthen the suggestion of the involvement of this multisubstrate docking site in Tie 2-mediated endothelial cell sprouting [Jones *et al.*, 1999]. However, the ligand(s) for Tie 1 remain unknown at present and no angiopoietin has been described that binds to Tie 1 [Hanahan, 1997].

The creation and characterization of mice in which either Tie 1 gene or Tie 2 gene has been disrupted have demonstrated that these two RTKs are not functionally equivalent and that each form plays a unique role in embryonic vasculogenesis

and/or angiogenesis. Deficiency of Tie 1 gene and Tie 2 gene *in vivo* results in embryonic lethality due to abnormalities of blood vessel formation during embryogenesis [Dumont *et al.*, 1994; Sato *et al.*, 1995]. Tie 2 knockout mice die during embryogenesis at day 9.5 and 10.5 because blood vessels are immature and lack branching networks, capillary sprouting from the primitive vascular network, and proper organization into large and small vessels, as observed in Ang-1 null mice and transgenic mice overexpressing Ang-2. The observations in these Tie 2 null mice have been interpreted as evidence for the role of Tie 2 system in regulating the endothelial cell recruitment of stromal cells which are required to encase and to stabilize the structure of blood vessels. Thus the Ang-1/Tie 2 pathway has a critical involvement in embryonic vascular development. Mice deficient in Tie 1 display embryonic lethality between day 14.5 and birth as a result of edema and hemorrhage. The finding of edema and hemorrhage in these mice can be attributed to poor structural integrity of the endothelial cells and firmly establishes the role of Tie 1 signaling in modulation of the hemodynamics of transcapillary fluid exchange. Based on the above-mentioned knockout phenotype, Tie 1 is thought to act as a regulator of angiogenesis and vascular maturation [Loughna *et al.*, 2001].

Although both angiopoietins and VEGF have been observed to regulate angiogenesis *in vitro* and *in vivo*, their roles in vascular polarity have not yet been fully elucidated. The molecular and genetic basis for vascular polarity should be investigated since there is a differential effect on right-hand side (RHS) and the left-hand side (LHS) venous formation system in double-knockout mouse embryos for Ang-1 and Tie 1. Their RHS venous formation is disrupted, whereas that is normal for LHS venous network. A possible explanation for this observation is that polarized expression of Ang-1 in combination with the Tie 1 signaling pathway(s)

only contributes to the formation of the RHS venous network rather than the LHS network. Distinct signal transduction pathways exist between the RHS and the LHS venous systems during their early stages of formation. Identification of functional ligand(s) for the Tie 1 receptor would be important for the investigation of the mechanism of left-right asymmetry of the venous development system.

The remodeling of blood vessels at the fetal-maternal interface and the formation of the placental fetal vascular tree have been studied based on the expression of Tie 2 receptor in fetal and maternal endothelial cells and endovascular invasive trophoblasts, and the expression of the ligand Ang-2 in syncytiotrophoblast [Goldman-Wohl *et al.*, 2000]. Ang-2 produced by the fetal syncytiotrophoblast migrates to both fetal and maternal endothelial cells where Tie 2 is expressed, due to the ligand-receptor pair Ang-2 and Tie 2. The primary function of an appropriately timed vascular remodeling is to sustain the pregnancy.

The pathological effects of Tie 1 and Tie 2 receptor tyrosine kinases include synovial proliferation and angiogenesis of rheumatoid arthritis [Uchida *et al.*, 2000] and breast-cancer angiogenesis by Tie 2/angiopoietin system [Stratmann *et al.*, 2001]. Rheumatoid arthritis (RA) is mainly characterized by angiogenesis [Uchida *et al.*, 2000]. Using immunohistochemistry and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis, it was found that both Tie 1 and Tie 2 receptors were expressed not only in vessel walls but also in synovial lining cells, stromal cells and pericytes. There is also a differential distribution pattern of Tie 1 expressing cells and Tie 2 expressing cells in synovial lining and stromal cells. The expression of these receptors reflects the degree of vascularity and synovial proliferation. The restricted expression of Tie 2 in CD69-/vimentin+ cells provides strong evidence for the role of Tie 2 in the differentiation of these cells from fibroblastic phenotype.

There should be the direct involvement of Tie 2 in the proliferation of lining cells, as demonstrated in the overexpression of PCNA in Tie 2 positive basal layered cells. The possible mechanisms of Tie receptors may be their interaction with the secreted angiopoietins in the endothelium itself or in the surrounding undifferentiated cells for further growth of the vessels, following the differentiation of CD34 positive endothelial progenitor cells into endothelial cells within synovial tissue from peripheral blood. In fact, the expression of Tie 1 and Tie 2 receptors in the pathologically abnormal synovial tissue of RA shed some light on their possible functional involvement in angiogenesis and proliferation of RA synovium.

Similar to the previously mentioned human gastric carcinoma (Section 1.32) and other types of cancer, breast cancer is known to be angiogenesis-dependent [Stratmann *et al.*, 2001]. The expression patterns of VEGFR-2 and Tie 2 in the human carcinoma samples suggest their biologic importance in tumour-associated angiogenesis. VEGFR-2 expression was mainly detected in endothelial cells in the tumour stroma and in small vessels within tumour islets, whereas there is a preferential expression of Tie 2 in endothelial cells in stromal vessels. Tie 2/Ang-1 signaling pathway may also be partly responsible for breast tumour growth. This is supported by the abundant expressions of both Tie 2 and Ang-1 in a murine breast-cancer cell line M6378 tumours as well as in tumours with marked hypoxia-inducible VEGF expression. The involvement of Ang-1 in tumour-associated angiogenesis reveals its functions in supporting the association of endothelial cells with smooth muscle cells and pericytes and in vascular sprouting. In contrast, reduction of both Tie 2 and VEGFR-2 would inhibit the growth of M6378 tumours.

1.6 Angiopoietin expression in female reproductive tissues (ovary)

Recently, the mechanism of angiopoietin action in the angiogenesis of the female reproductive system has become the focus of intense interest. In female adults, physiological angiogenesis is of particular significance in follicular development, corpus luteum formation, and uterine endometrial proliferation during the ovarian cycle. Recent studies demonstrated Ang-1 and Ang-2 mRNA expression in the ovary of rat [Maisonpierre *et al.*, 1997], cow [Goede *et al.*, 1998] and monkey [Hazzard *et al.*, 1999; Hazzard *et al.*, 2000].

Ovaries from gonadotrophin-induced ovulating rats have been selected as targets and angiopoietin expression was compared with VEGF RNA expression during vascular remodeling in the rat ovary [Maisonpierre *et al.*, 1997]. Using *in situ* hybridization approach, the mRNA expression of VEGF, Ang-1 and Ang-2 was localized in sections corresponding to four successive developmental stages: a small vesicular follicle, a large preovulatory follicle, a nonproductive follicle undergoing atretic regression and a developing corpus luteum at 8 h after ovulation. VEGF and Ang-2 expression were upregulated in the developing corpus luteum before vessel invasion. There were also abundant VEGF and Ang-2 transcripts at the front of vessels invading the developing corpus luteum. A possible scenario therefore was that the collaboration of Ang-2 with VEGF at the front of invading vascular sprouts enabled the endothelial cells to revert to a more plastic state so that the blood vessels would become more responsive to angiogenic changes induced by simultaneously available angiogenic factors such as VEGF. In agreement with a later role of Ang-1 than VEGF in angiogenesis, Ang-1 mRNA expression was observed to follow or coincide with, rather than precede, vessel ingrowth into the early corpus luteum. The autocrine induction of Ang-2 blocked the constitutive stabilizing influence of

paracrine Ang-1. The atretic follicle and aged corpus luteum were characterized by uniformly high expression of Ang-2, with subsequent vascular regression under the absence of VEGF expression. High Ang-2 levels in the absence of a strong VEGF induction suggested a destabilization of blood vessels that finally led to vessel regression.

Goede *et al.* (1998) investigated the molecular mechanisms underlying the processes of blood vessel maturation in the cyclic midstage bovine ovarian corpus luteum. The relative expression of Ang-2 over Ang-1 was examined in corpus rubrum mid-stage corpora lutea (CL), and regressing CL using quantitative PCR-based technique. Results from this study demonstrated similar mRNA levels for Ang- 1 and Ang-2 in angiogenic corpus rubrum as well as in the nonangiogenic corpus luteum. In contrast, the relative expression of mRNA for Ang-2 to Ang-1 was up-regulated during vessel regression when compared to that in the corpus rubrum and in the mid-stage CL. This would be in agreement with the previously published studies of Maisonpierre *et al.* (1997), which also showed the relative overexpression of Ang-2 during blood vessel regression.

Hazzard and his co-workers (1999) were the first to report on time-specific angiopoietin expression during the ovarian cycle in mono-ovular species, primates. As one of the pilot experiments in this study, the time-dependent effect of recombinant human gonadotrophins (rhCG) in inducing Ang-1 and Ang-2 mRNA expression was examined in peri-ovulatory follicles at a final concentration of 1000 IU. hCG was shown to induce a dramatic increase (30-fold) in Ang-1 mRNA levels in granulosa cells at 36 h hCG treatment (around ovulation), an event that closely followed a 10-fold decrease in Ang-1 mRNA levels at 12 h after hCG administration. This would be expected since Ang-1 production is augmented to stabilize vasculature

by recruiting pericytes to developing capillaries after the breakdown of the basement membrane close to the time of ovulation. In contrast, no changes in Ang-2 mRNA levels were observed in primate granulosa cells of periovulatory follicles at 12 or 36 hours after hCG administration. This might reflect the constant expression of Ang-2 in the maturing follicle during the peri-ovulatory interval. Such observation was in line with the decline of Ang-1 mRNA expression at 12 hours after hCG treatment and could be related to the initiation of plasticity increase and loosening of the capillary support matrix during angiogenesis.

In the study of gonadotrophin and steroid regulation of Ang-1 and Ang-2 mRNA expression during the peri-ovulatory interval, one group of monkeys received 3β-HSD inhibitor Trilostane (TRL) treatment. For the restoration of progestin concentration, another group of monkeys received TRL plus the non-metabolizable progestin R5020 (Promegestrone) treatment. The effects were examined following 12- and 36-hour treatment periods and compared with the above-mentioned effects of hCG administration (without any additional treatment) at each time point for both groups of animals. The non-responsiveness of Ang-1 mRNA expression to TRL or TRL+R5020 at 12 h after hCG treatment was related to the direct regulation of Ang-1 mRNA expression by hCG rather than progesterone around the time of ovulation. Although the up-regulation of Ang-1 was significantly overcome by the inhibitory effect of TRL in the late peri-ovulatory interval, a partial restoration of Ang-1 mRNA to time-matched control levels by progestin occurred at 36 h after hCG treatment. The observation would suggest that there was a gonadotrophin-dependent regulation of Ang-1 mRNA expression during the early stage of peri-ovulatory period but a steroid-dependent regulation during the late peri-ovulatory stage (at 36 h after hCG treatment). In sharp contrast to Ang-1 expression, the inhibitory effect of

TRL on Ang-2 expression was observed at 12 h after hCG treatment but Ang-2 mRNA levels could not be brought to control values by progestin replacement. Also, neither TRL nor TRL+R5020 were shown to exert any effects on Ang-2 expression at 36 h after hCG treatment. It was likely that Ang-2 expression was regulated by steroid during the peri-ovulatory interval (at 12 h after hCG treatment) rather than during the late-ovulatory period (at 36 h after hCG treatment), as indicated by the lack of progestin effect at 12 h after hCG treatment.

More recently, Hazzard *et al.* (2000) demonstrated the expression of Ang-1 and Ang-2 mRNA in the primate corpus luteum throughout the luteal lifespan in the menstrual cycle. Corpus luteum was collected from rhesus monkeys at different time points. The time intervals chosen for this study were early- (3-5 days post-LH surge), mid- (6-8 days), mid-late (10-12 days), and late (14-16 days) luteal phase of the menstrual cycle, and at menstruation (17-18 days). Ang-1 and Ang-2 mRNA expressions during different stages of the luteal lifespan in the menstrual cycle were assessed by semi-quantitative RT-PCR approach. The possible association of Ang-1 with its natural antagonist Ang-2 in facilitating the development and regression of vasculature in the primate corpus luteum was characterized by their similar expression patterns during the luteal phase. Both Ang-1 and Ang-2 mRNA levels remained low at early to mid-luteal phase, peaked during corpus luteum regression at late luteal phase and then declined towards the basal by the time of menstruation.

The results obtained by Goede *et al.* (1998) appear to be totally different from those obtained from Hazzard *et al.* (2000). Similar expression patterns of Ang-1 and Ang-2 during the luteal phase were observed in the study carried out by Hazzard *et al.* (2000), whereas Goede *et al.* (1998) demonstrated an up-regulation of the relative expression of mRNA for Ang-2 to Ang-1 in the late luteal phase in the cow. Goede

et al. (1998) realized that these observations could be explained simply by the regression of capillaries at late luteal phase. Hazzard *et al.* (2000) disagreed with such observations and suggested a new concept of compartmentalized expression of Ang-1 and Ang-2. This concept was supported by the localization of Ang-1 and Ang-2 mRNA in different areas of the mouse corpus luteum using *in situ* hybridization by Maisonpierre *et al.* (1997). According to this revised concept, the decrease in functional interaction among these angiogenic factors might influence vascular development or regression. In addition, based on an *in vitro* study performed by Mandriota & Pepper (1998), there was evidence for the inhibition of Ang-2 expression by angiogenic repressors in bovine microvascular endothelial cells under the presence of Ang-1. Nevertheless, it remains unclear whether the expression patterns of Ang-1 and Ang-2 during the luteal phase are species-specific or whether the stimulation of gonadotrophins, mainly LH/hCG, is involved in the regulation of VEGF mRNA levels and subsequent Ang-1 and Ang-2 expressions in the ovarian follicles and corpora lutea.

1.7 Testicular angiogenesis

There have been very few studies that identify the regulators of testicular angiogenesis and establish the molecular mechanisms underlying angiogenesis in testis under physiological and pathological conditions. Using RT-PCR and immunohistochemical staining, VEGF transcripts and proteins have been localized mainly to Leydig cells and Sertoli cells in the testes of both human [Ergun *et al.*, 1997] and rodents species [Wan *et al.*, 1996; Collin *et al.*, 1996; Au *et al.*, 1997]. It has also been proposed that besides the possible involvement of VEGF in testicular

angiogenesis, VEGF may play an important role in Leydig cell-testicular macrophage interaction [Au *et al.*, 1998]. Using a cell migration assay, VEGF has been shown to induce chemotactic movement of testicular macrophages suggesting that VEGF produced by Leydig cells could be involved in the recruitment of testicular macrophages, allowing the two cell types to establish a close structural and functional association [Hutson, 1998].

Since the process of angiogenesis involves not just VEGF and its receptors as previously thought but also the interaction of angiopioetins with their Tie 2 receptors. The interaction of the latters is important in determining the stability of the blood vessels either by stabilizing the endothelial cell interaction with the surrounding cells to maintain the structure of the blood vessels or to interfere with such interaction leading to either increased angiogenic potential or degeneration through apoptosis. Although VEGF and its receptors have previously been demonstrated in the testis where they occur in specific cell types, information of angiopoietins and Tie is entirely missing. In order to better understand the process of testicular angiogenesis and its possible control, it would therefore be interesting to determine whether and how the expression of angiopoietins is spatially related to the known distribution of VEGF in the testis.

1.8 Aims of the present study

Apart from VEGF and its receptors, in order to gain further insight into the possible involvement of angiopoietins and Tie in the control of testicular angiogenesis, the present study examined the expression and cellular localization of angiopoietins 1, 2, and 3, and their related receptor tyrosine kinases, Tie 1 and Tie 2

in the rodent testes and testicular cell lines using a combination of techniques. These include RT-PCR, Western immunoblotting and immunohistochemistry. The use of testicular cell lines from rat and mouse allows the possible sources of angiopoietins from the two major somatic cell types within testis to be differentiated. In addition, testicular tissues and primary testicular cells were used to confirm angiopoietin expression *in vivo* and to correlate with the findings obtained *in vitro* from testicular cell lines which may have changed their phenotypic expression after multiple passages in culture.

Also in view of the recent reports on the expression of multiple Ang-1 isoforms in the human megakaryocyte cell line CHRF [Huang *et al.*, 2000] and two Ang-2 isoforms from human umbilical vein endothelial cells [Kim *et al.*, 2000a], the present study was extended to determine whether such isoforms could be expressed in animal species such as the rat and mouse, and in particular for tissues such as the testis. Such possibilities were being examined in the present study using RT-PCR and specific primers that were designed based on information gathered from the study of human.

2. Materials and methods

2.1 Preparation of primary cells from rat testes

2.1.1 Sertoli cell preparation

Sertoli cells were prepared from 20-day-old Sprague-Dawley rats following the methods described by Gorczynska *et al.* (1993) and Gnessi *et al.* (1995) with some modificiations. The animals were killed by carbon dioxide asphyxiation. Testes were excised using sterile instruments and placed into 10 mL sterile Hanks' balanced salt solution containing 0.01% of deoxyribonuclease (#DN25, Sigma, St. Louis, MO, USA) (HBSS-DNase). Subsequent procedures were carried out in a laminar flow hood using aseptic technique. After rinsing with HBSS-DNase, the testes were decapsulated and the contents transferred to 5 mL fresh HBSS-DNase. The seminiferous tubules were teased apart and chopped into small pieces for 15 minutes. The minced tissue was transferred into a sterile 50 mL-Falcon tube and washed twice in HBSS-DNase made up to a total volume of 50 mL. Every time after washing, the tissue was recovered by centrifugation at 250 g for 3 minutes, and the supernatant was discarded. The tubule fragments were then digested in 10 mL HBSS containing 0.01% DNase, 0.1% trypsin (#T8003, Sigma, St. Louis, MO, USA), 0.1% hyaluronidase (#H2251, Sigma, St. Louis, MO, USA) and 0.01% collagenase (#C9891, Sigma, St. Louis, MO, USA) in a shaking water bath (180 cycles per minute) at 32 $^{\circ}$C for 25 minutes. After digestion, the tissue was transferred to a sterile 50 mL-Falcon tube and again washed twice in HBSS-DNase in a final volume of 50 mL. The supernatant was discarded each time after centrifugation at 250 g for 3 min at room temperature. At the end, the tissue pellet was resuspended in 12 mL Dulbecco's Modified Eagle Medium: Ham's F12 medium (DMEM/F-12, 1:1, v/v)

(Gibco BRL, Grand Island, NY, USA, Cat. No. 12500-062) supplemented with 10% fetal bovine serum (Gibco BRL, Grand Island, NY, USA), 5 μg/mL human transferrin (#T8158, Sigma, St. Louis, MO, USA), 10 μg/mL bacitracin (#B0125, Sigma, St. Louis, MO, USA), 2.5 ng/mL epidermal growth factor (#E4127, Sigma, St. Louis, MO, USA) and 10 μg/mL bovine insulin (#I5500, Sigma, St. Louis, MO, USA). The cells were plated onto six sterile culture plates (100 x 20 mm; Falcon, Becton Dickinson Labware, Franklin Lakes, NJ, USA) and placed in a water-jacketed carbon dioxide incubator (Napco Model 5100) maintained at 32 $^{\circ}$C under a humidified atmosphere of 5% carbon dioxide in air. The medium was changed the next day. On the 2nd day of culture, the cultures were subjected to hypotonic treatment in 20 mM TRIS (pH 7.4) for 2.5 minutes to lyse the residual germ cells. This was then followed by three washes with DMEM/F-12. On the 4th day of culture, the medium was aspirated and the cells that remained in the culture dish represented an enriched Sertoli cells preparation. They were ready for RNA or protein extraction.

2.1.2 Germ cell preparation

Germ cells were isolated from testes of 90-day-old male Sprague Dawley rats using the method described by Woodruff *et al.* (1992) with minor modifications. The testes were decapsulated and the tissue was transferred to a clean milk bottle. Twelve millilitres of DMEM/F-12 containing 30 mg bovine serum albumin (#A3294, Sigma, St. Louis, MO, USA) and 3 mg collagenase (Type I; Worthington Biochemical Corporation, Halls Mill Road, Freehold, New Jersey) were added and after replacing the screw cap, the bottle was tightly sealed

with parafilm. The digestion process was carried out in a shaking water bath (110 cycles per minute) at 32 °C for 20 minutes. At the end, the tubules were dispersed in 50 mL DMEM/F-12 and shaken gently. Then the tissue was allowed to sediment for 5 minutes and the supernatant was discarded. The tubules were further washed three times with 100 mL 10 mM phosphate-buffer saline (PBS, pH 7.4, containing 9 g/L sodium chloride and 1.56 g/L sodium dihydrogen phosphate monohydrate) and shaken gently. Each time the tissue was allowed to sediment for 10 minutes and the supernatant was discarded each time. The resulting tubules were thoroughly minced with scissors for 10 minutes and the minced tissue was transferred to a clean 50 mL-Falcon tube. PBS was then added to the tube to make up the final volume to 50 mL. After gentle shaking, the chucks of tissue fragments were pelleted by centrifugation at 6 g for 2 minutes. The supernatant collected after centrifugation was allowed to pass through a nylon pantyhose to remove aggregated cells and larger debris. The above steps were repeated two more times with the tissue pellets remaining at the bottom of the centrifuge tube. The resulting filtrate was added to a funnel packed with glass wool to remove the spermatozoa and then centrifuged at 100 g for 4 minutes. The supernatant was discarded and the pellet at the bottom of the tube was washed twice with 50 mL PBS. At the end, the pellet was resuspended in 10 mL PBS and the cell suspension was filtered through a 20 μm nylon mesh. The final filtrate was centrifuged at 1500 g for 5 minutes. After pipetting off the supernatant, the cell pellet containing predominantly germ cells was ready for RNA or protein extraction.

2.1.3 Interstitial cell and Leydig cell preparation

The procedures used for preparing interstitial cells and Leydig cells were adopted from Murono *et al.* (1992) and Gnessi *et al.* (1995). Both cell preparations were derived from testes of 90-day-old adult Sprague Dawley rats. Testicular interstitial cells were obtained after collagenase digestion of the testes followed by crude cell separation by centrifugation. Leydig cells were obtained after subjecting the interstitial cells to a further purification step of Percoll density gradient centrifugation.

After the animals were killed by carbon dioxide asphyxiation, their testes were removed and decapsulated. The tissue was placed in a milk bottle containing 24 mL or 12 mL 0.025% collagenase (Type I; Worthington Biochemical Corporation, Halls Mill Road, Freehold, New Jersey) solution in Medium 199 (Gibco BRL, Grand Island, NY, USA; Cat. No. 21200-076) or DMEM/F-12 supplemented with 60 mg BSA, 10 units/mL penicillin G sodium and 10 μg/mL streptomycin sulphate (Gibco BRL, Grand Island, NY, USA) for the preparation of Leydig cells and interstitial cells, respectively. Enzyme digestion was allowed to proceed in a shaking water bath (110 cycles per minute) at 32 $^{\circ}$C for 20 minutes. At the end, 80 mL DMEM/F-12 supplemented with antibiotics and 0.2% BSA (for interstitial cell preparation) or 50 mL Medium 199 supplemented with antibiotics (for Leydig cell preparation) was added to the milk bottle and the testicular tissue was allowed to sediment for 5 minutes. The supernatant was pipetted off to 50 mL-Falcon tubes. For preparing Leydig cells, 25 mL Medium 199 was again added to the tubes. Similarly, for preparing interstitial cells, DMEM/F-12 with 0.2% BSA was added to the tubes to make up the final volume to 50 mL. The cells were collected in the supernatant after

gently swirling and unit sedimentation.

After the above steps, the supernatant was pooled and centrifuged at 350 g for 10 minutes. For preparing interstitial cells, 10 mL PBS was added to wash the cell pellet twice. The tubes were centrifuged at 350 g for 5 minutes and the supernatant was then discarded each time. The cell pellet was finally ready for protein extraction.

For preparing Leydig cells, the cell pellet was recovered and resuspended in a small volume of Medium 199: BSA (50 mL of Medium 199 supplemented with 10 units/mL penicillin G sodium, 10 μg/mL streptomycin sulphate and 50 mg BSA). The cells were pooled and layered on top of a discontinuous Percoll (Amersham Pharmacia Biotechnology, Buckinghamshire, UK) gradient with density of 1.030, 1.040, 1.055, 1.065, 1.070 and 1.096 g/L set up in 50 ml Falcon centrifuge tube. The gradient was centrifuged at 900 g for 20 minutes. Cells localizing at percoll gradient density of 1.070 g/L were saved as the Leydig cell enriched fraction. Medium 199: BSA (50 mL of Medium 199 supplemented with 10 units/mL penicillin G sodium and 10 μg/mL streptomycin sulphate and 50 mg BSA) was added to wash the Leydig cells three times, with centrifugation at 350 g for 10 minutes in between each washing step. The resulting Percoll purified Leydig cells were then ready for RNA extraction. In view of the large number of animals required to get enough purified Leydig cells for protein analysis, Western immunoblotting was not performed on Leydig cell lysates, but rather just on the interstitial cell lysate.

2.2 Cell cultures

2.2.1 Reagents and cell lines

Testicular cell lines (TM3, TM4, MLTC-1, R2C and LC540), and rat C6 glioma cell line were obtained from the American Type Culture Collection (Rockville, MD) and maintained under recommended conditions. 1:1 Dulbecco's Modified Eagle Medium (DMEM):Ham's F12 Medium (Cat. No. 12500-062), RPMI 1640 medium buffered with 25 mM HEPES (Cat. No. 23400-021), Ham's F10 medium (Cat. No. 81200-040), Minimum Essential Medium (Eagle) in Earle's BSS (Cat. No. 41500-067), fetal bovine serum, horse serum, penicillin G (sodium salt) and streptomycin sulphate were purchased from Gibco BRL, Grand Island, New York, USA. Sodium bicarbonate and D-glucose were obtained from BDH Chemicals Ltd, Poole England. HEPES (without sodium salt) was purchased from Boehringer Mannheim, Germany. Tissue culture plates (100 x 20 mm; Polystyrene; Nonpyrogenic) were from Becton Dickinson Labware, Becton Dickinson and Company, Franklin Lakes, NJ, USA.

2.2.2 Cell lines of mouse TM3 Leydig cells and TM4 Sertoli cells

Based on information provided by the supplier (ATCC), both TM3 Leydig cells and TM4 Sertoli cells are clonal cell lines isolated respectively from primary cultures of Leydig cell-enriched preparations and Sertoli cell-enriched preparations from normal testes of 11- to 13-day-old BALB/c nu/+ mice. They were maintained in a 1:1 mixture of Dulbecco's Modified Eagle Medium (DMEM)/Ham's F12 medium supplemented with 4.5 g/L glucose, 1.2 g/L sodium bicarbonate, 15 mM HEPES, 10 units/mL penicillin G (sodium salt) and 10 μg/mL streptomycin sulphate, 5% horse serum and 2.5% fetal bovine serum.

The cultures were kept at 37°C in a water-jacketed CO_2 incubator (Nuaire, US Autoflow, Techcomp) and media were changed every 3 days. The cells were passaged at a dilution ratio of 1:5 after a period of 3-5 days and harvested at 80-90% confluence.

2.2.3 Mouse MLTC-1 Leydig tumour cells

According to the information provided by the supplier, MLTC-1 is a tumour cell line of mouse Leydig cells derived from the M548OP transplantable Leydig cell tumour carried in C57BL/6 mice. Significant stimulation of membrane adenyl cyclase activity could be observed in these cells after treatment with human chorionic gonadotrophin (hCG), luteinizing hormone, cholera toxin, sodium fluoride and guanyl-5'-ylimidodiphosphate. The cells were cultured in RPMI 1640 medium containing 25 mM HEPES, 10% fetal bovine serum, 10 units/mL penicillin G sodium and 10 μg/mL streptomycin sulphate at 37 °C under a humidified atmosphere of 5% CO_2 in air. The medium was replaced every 3 days and the cells were passaged at a split ratio of 1:3 at 5-6 days intervals.

2.2.4 Rat R2C Leydig tumour cells

A tumour cell line of rat Leydig cells (R2C) is derived from testes of two-month-old male rat. The cells were cultured in Ham's F10 medium supplemented with 15% horse serum, 2.5% fetal bovine serum, 10 units/mL penicillin G sodium and 10 μg/mL streptomycin sulphate but the medium for culture of rat glial cells (C6) had additional supplements of 1.5 g/L sodium bicarbonate and 2 mM L-glutamine. The cells were kept at 37 °C in a humidified 5% CO_2 atmosphere and the cells were fed every 3 days. They were passaged

upon confluency at a split ratio of 1:3 at 3-5 days intervals.

2.2.5 Rat LC540 Leydig tumour cells

LC540 Leydig cells are derived from Fischer male adult rat. They were grown in Minimum Essential Medium (Eagle) in Earle's BSS supplemented with non-essential amino acids, 1 mM sodium pyruvate and 10% fetal bovine serum. The medium also had 10 units/mL penicillin G sodium and 10 μ g/mL streptomycin sulphate added as antibiotics. The cells were kept in a humidified 5% CO_2 atmosphere at 37 °C, and the medium was changed every 3-4 days. The cells were passaged at a split ratio of 1:3 after a period of 3-5 days.

2.2.6 Rat C6 glioma cells

According to information provided by the supplier and other published studies, C6 is a tumour cell line of rat glial cells (C6). The medium for culture of this cell line was the same as that for culture of rat R2C Leydig cells, except that it had additional supplements of 1.5 g/L sodium bicarbonate and 2 mM L-glutamine. The cells were kept at 37 °C in a humidified 5% CO_2 atmosphere and the cells were fed every 3 days. They were passaged upon confluency at a split ratio of 1:3 at 3-5 days intervals.

2.3 Analyses of Angiopoietin 1, Angiopoietin 2, Angiopoietin 3, Tie 1 receptor, and Tie 2 receptor mRNA in testicular cell lines and testicular tissues

2.3.1 Extraction of total RNA from testicular cell lines and testicular tissues

Total RNA was isolated from testicular cell lines and testicular tissues using TRIzol reagent following the procedure suggested by the manufacturer (Gibco BRL, Grand Island, NY, USA). One mL TRIzol reagent was used for 50-100 mg testicular tissue. After the addition of TRIzol reagent, the tissue was hand-homogenized in a 3-mL glass homogenizer with a clearance of 0.004-0.006 inch (Kontes Scientific Glasswarer/ Instruments, Vineland, NJ, USA). During tissue homogenization, the homogenzier was kept cooled inside an ice bucket. The tissue lysate was centrifuged at 12,000g for 10 minutes at 4 °C in an Eppendorf Refrigerated Centrifuge (Eppendorf 5804R, Hamburg, Germany) and the resulting clear supernatant was transferred to a new 1.5 mL microcentrifuge tube.

Testicular cell lines grown in monolayer were lysed directly in culture dishes by adding 1 mL TRIzol reagent to each 10 cm^2 of culture. Subsequently the cell suspension was passed several times through a 1 mL syringe to disrupt the cells.

Phase separation was carried out for both tissue lysate and cell lysate. The homogenized samples were incubated at room temperature for 15 minutes to permit complete dissociation of nucleoprotein complexes. 200 µL of chloroform was added per 1 mL of TRIzol reagent initially used for homogenization. The

sample tubes were capped securely and then hand-shaken vigorously for 15 seconds. After further incubation of the samples at room temperature for 3 minutes they were subjected to centrifugation at 12,000 g for 15 minutes at 4 °C. Following centrifugation, the mixture was separated into a lower red, phenol-chloroform phase, an interphase, and a colourless upper aqueous phase. The upper aqueous phase containing RNA was then transferred into a new 1.5 mL microcentrifuge tube. RNA was precipitated by the addition of 500 µL isopropyl alcohol per 1 mL of TRIzol reagent used for the initial homogenization and then the samples were incubated at room temperature for 10 minutes. The samples were subjected to centrifugation at 12,000g for 10 minutes at 4 °C and RNA should be visible on the side and bottom of the tube. The supernatant was then discarded and the RNA pellet was washed once with 1 mL 70% ethanol per 1 mL of TRIzol reagent used initially for homogenization. This was done by vortexing the microcentrifuge tube to dislodge the pellet and then collecting the pellet again after centrifugation at 7,500g for 5 minutes at 4 °C. The supernatant was carefully discarded and the RNA pellet was vacuum-dried briefly for 5 to 10 minutes using the SpeedVac (DNA SpeedVac 110, Savant Instruments Inc., NY, USA) to remove residual ethanol. Nontheless the pellet should not be overdried since this would make the redissolving more difficult. The RNA was dissolved in an appropriate volume of diethylpyrocarbonate (DEPC)-treated water and the solution was passed a few times through a pipette tip to facilitate dissolution. The resulting RNA samples could then be stored at –70 °C until use.

2.3.2 Quantitation of total RNA

RNA concentrations and purity were determined by spectrophotometry (Ultrospec Spectrophotometer, Amersham Pharmacia Biotechnology, Buckinghamshire, UK) based on the absorbance at 260 nm (A_{260}) and 280 nm (A_{280}). Each RNA sample was diluted a hundredth time before being placed in a curvette which had previously been rinsed with DEPC-treated water to remove any contaminants or residual RNA/DNA from previous samples. DEPC-treated water was used as a reagent blank to compensate for the loss of light caused by reflections from the surface of the cuvette and the absorbance of the solvent (i.e. DEPC-treated water). RNA concentration, total yield and the purity of each sample could then be calculated according to the following formulae:

RNA concentration of original sample (in μg per μL) = (40 x A_{260} x dilution factor) / 1000

Total yield (in μg) = RNA concentration x total volume of RNA sample (in μL)

RNA purity = A_{260} / A_{280}

An absorbance of 1 unit at 260 nm corresponds to 40 μg of RNA per mL (A_{260} = 1 = 40 μg per mL) and this relationship is valid only for measurements in water. Absorbance readings at 260 nm measure RNA concentration and they should be greater than 0.15 to ensure significance. The ratio of absorbance readings taken at 260 nm and 280 nm (A_{260} / A_{280}) provides an estimate of the purity of the RNA sample. Only when this ratio lies between 1.5 and 1.9 would the RNA preparation be considered acceptably pure. When this ratio is lower than 1.5, the RNA is considered contaminated (with protein).

2.3.3 First strand cDNA synthesis by reverse transcription (RT)

Reverse transcription was firstly performed by heating a 12 μL reaction mixture containing 5 μg total RNA and 0.5 μg/μL oligodeoxythymidine primers (Gibco BRL, Grand Island, NY, USA) to 70 °C for 10 minutes. After immediate chilling of the sample in ice for 2 minutes, 7 μL mixture containing 5x first strand buffer, 0.1 M dithiothreitol (DTT) and 10 mM each of the deoxynucleotidetriphosphates (dNTPs) (Gibco BRL, Grand Island, NY, USA) was added to each RNA sample and then heated to 42 °C for 2 minutes. 1 μL of Superscript II RNase H⁻ Reverse Transcriptase (200 units) (Gibco BRL, Grand Island, NY, USA) was then added to each sample in a final volume of 20 μL and incubated for 50 minutes at 42 °C, followed by a final extension time of 15 minutes at 72 °C. All cDNA preparations were kept at –20 °C until use for RT-PCR.

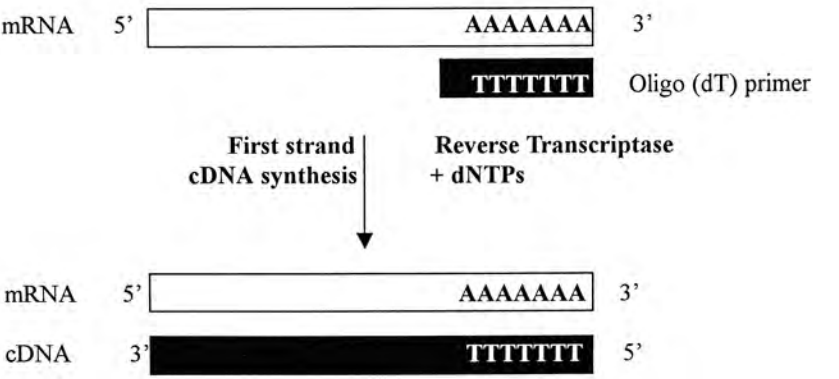


Figure 2.1 Schematic drawing showing first strand cDNA synthesis by reverse transcription

2.3.4 Normalization of the amounts of cDNA used in polymerase chain reaction (PCR)

The amounts of cDNA used in polymerase chain reactions (PCR) were adjusted based on a house-keeping gene – β-actin. Amplification of cDNA for β-actin serves to verify successful RNA isolation, reverse transcription from total RNA to cDNA and equal loading of cDNA in PCR.

One fiftieth of synthesized cDNA was used as a template in the PCR reaction for β-actin containing 2 μL 10x PCR buffer, 1 μL 10 mM each dNTP, 1 μL each of sense and antisense primers (20 pmoles per μL), 0.5 μL Taq DNA polymerase (Gibco BRL, Grand Island, NY, USA) and 2.0 mM magnesium chloride in a final volume of 20 μL. Samples were pre-heated to 94 °C for 3 minutes, followed by 18 PCR cycles (94 °C for 1 minute, 58 °C for 45 seconds, 72 °C for 1 minute 30 seconds) and a final extension step at 72 °C for 10 minutes, using a PTC 200 thermal cycler (Thermal Cycler, PTC-200, MJ Research, San Fancisco, USA). Based on earlier studies, the number of cycles (i.e. 18 cycles) chosen for the amplification of β-actin cDNA was found at the exponential phase of the PCR. The following primer sequences specific for β-actin were selected:

sense primer 5'-TCACCGAGGCCCCTCTGAACCCTA-3'

antisense primer 5'-GGCAGTAATCTCCTTCTGCATCCT-3'

They correspond to sequences 1642-1665 bp and 2814-2837 bp, respectively (GenBank™ Accession Number J00691), and should give rise to a PCR product with the expected size of 645-bp. All primers used in the present study were purchased from Gibco BRL, Grand Island, NY, USA. 10 μL of each PCR reaction mixture were analyzed by electrophoresis in 1.2% agarose gel

(electrophoresis grade, Gibco BRL, Grand Island, NY, USA) containing 0.5 mg/mL ethidium bromide in TAE buffer (40 mM TRIS, 20 mM sodium acetate, 1 mM EDTA, pH 7.2). The position and relative intensities of the bands were recorded and compared using a gel documentation system (Fluorchem™ 8000 Advanced Fluorescence, Chemiluminescence and Visible Light Imaging System, Alpha Innotech Corporation). Based on the difference in band intensities for β-actin, the amounts of templates used in subsequent PCR reactions for the transcripts of special interest were adjusted accordingly to approximate equal loading.

2.3.5 Polymerase chain reaction (PCR)

Before performing the PCR for specific gene products the reaction conditions were optimized for primer annealing temperature, concentration of magnesium chloride, PCR cycle number and initial amounts of cDNA template used for each primer set. A series of RT-PCR experiments was then conducted using pairs of specific primers for Ang-1, Ang-2, Ang-3, Tie 1 and Tie 2. Table 2.1 summarizes the primer sequences designed from published DNA sequences whereas Table 2.2 summarizes the determined optimal primer annealing temperature, optimal concentration of magnesium chloride, optimal PCR cycle number and the expected product size for each PCR.

PCR was performed in thin wall 0.6 mL PCR tubes (Robbins Scientific Corporation, San Aleso Avenue, Sunnyvale, CA) with variable amount of template, variable amount of 50 mM magnesium chloride, 2 μL of 10X PCR buffer, 1 μL of 10 mmoles each dATP, dCTP, dGTP and dTTP, 1 μL each of the

sense and antisense primers (20 pmoles), and 0.5 μL of 5 units per μL *Taq* DNA polymerase (Gibco BRL, Grand Island, NY, USA). Amplifications were carried out in 20-μL volumes using PTC-200 Thermal Cycler (M. J. Research, Inc., San Fancisco, USA) and the following parameters: 3 minutes 94 °C initial denaturation followed by optimal temperature cycles of denaturation at 94 °C for 1 minute, primer annealing for 45 seconds, and extension at 72 °C for 1 minute 30 seconds. The final cycle was followed by an additional extension at 72 °C for 10 minutes. The number of PCR cycles was 35 for Ang-3, Tie 1 and Tie 2. Master mixes were used whenever possible to reduce tube-to-tube variations.

For Ang-1 and Ang-2, a nested PCR approach was used in which the primary PCR product was used as the template for a second PCR using another pair of internal primers (or nested primers). For Ang-1, the PCR conditions were as follows:

(a) 1st PCR using S1 sense and AS3 anti-sense primers: 94 °C for 3 minutes followed by 25 cycles of 1 minute at 94 °C, 45 seconds at the primer annealing temperature of 60 °C, 1 minute 30 seconds at 72 °C, and a final extension of 10 minutes at 72 °C.

(b) 2nd PCR using S2 sense and AS2 anti-sense primers: 1 μL of primary PCR product was used as the template and heated to 94 °C for 3 minutes followed by 25 cycles of 1 minute at 94 °C, 45 seconds at the primer annealing temperature of 55 °C, 1 minute 30 seconds at 72 °C, and a final extension of 10 minutes at 72 °C.

For Ang-2, the PCR conditions were as follows:

(a) 1st PCR using S1 sense and AS1 anti-sense primers: 94 °C for 3 minutes followed by 19 cycles of 1 minute at 94 °C, 45 seconds at the primer annealing

temperature of 55 °C, 1 minute 30 seconds at 72 °C, and a final extension of 10 minutes at 72 °C.

(b) 2nd PCR using S2 sense and AS2 anti-sense primers: 1 μL of primary PCR product was used as the template and heated to 94 °C for 3 minutes followed by 25 cycles of 1 minute at 94 °C, 45 seconds at the primer annealing temperature of 58 °C, 1 minute 30 seconds at 72 °C, and a final extension of 10 minutes at 72 °C.

To examine the expression of Ang-1 isoforms in adult rat testis and several testicular cell lines, four sets of primers corresponding to different regions and isoforms of the Ang-1 nucleotide sequences were designed for a series of PCR experiments, based on the nucleotide and amino acid sequences of the coding region of the four Ang-1 isoforms published in Huang *et al.*, 2000, as shown in Table 2.3 and Table 2.4.

After PCR, aliquots of the reaction product (10 μL) were electrophoresed in 1.2% agarose gel containing 0.5 mg/mL ethidium bromide in TAE buffer (40 mM TRIS, 20 mM sodium acetate, 1 mM EDTA, pH 7.2).

Table 2.1 Primers used for the PCR amplification of Ang-1, Ang-2, Ang-3, Tie 1 and Tie 2

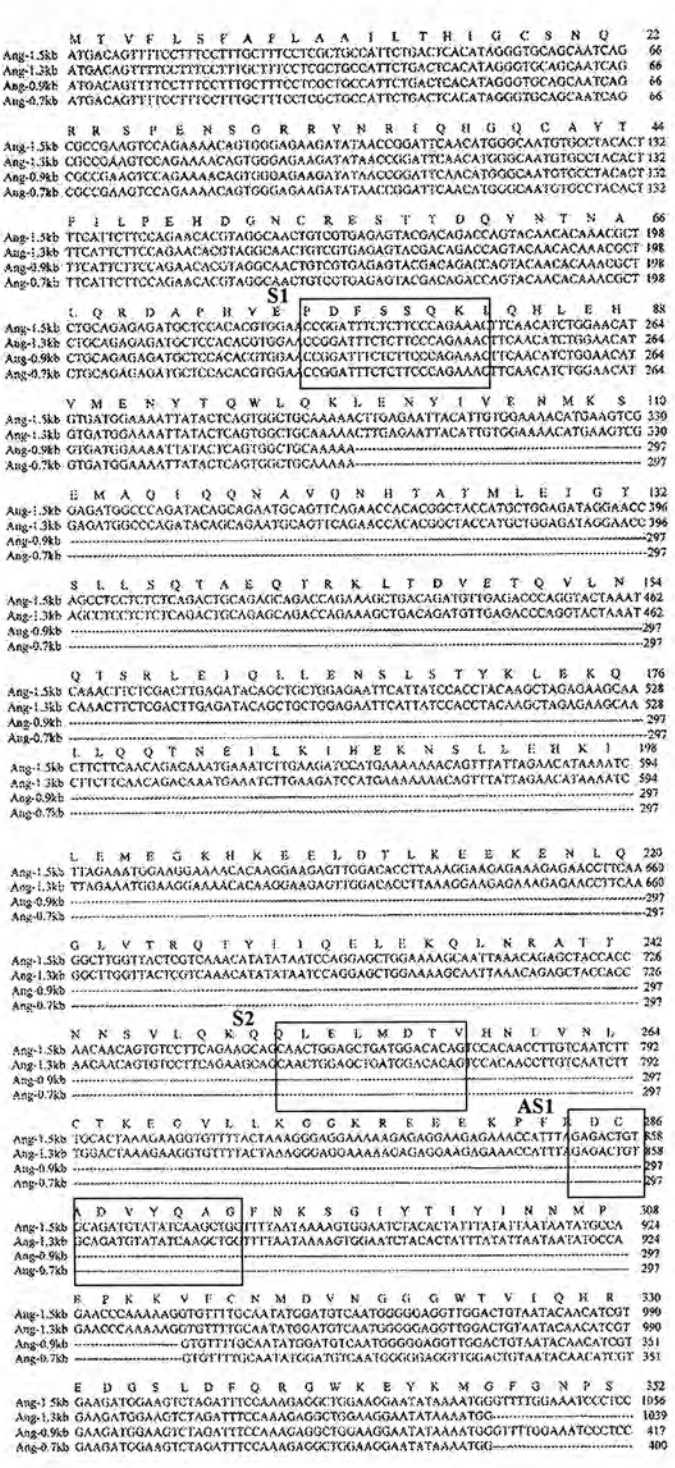
Target	Primer Sequences (5' to 3')
Ang-1	S1: CCG GAT TTC TCT TCC CAG AAA C (nt. 227-248) S2: CAA CTG GAG CTC ATG GAC ACA G (nt. 752-773) AS1: CCA GCT TGA TAT ACA TCT GCA CAG TCT C (nt. 1157-1184) AS2: AGC ATG TAC TGC CTC TGA CTG GT (nt. 1097-1119) AS3: CCT GCT GTC CCT GTG TGA CCT TTT (nt.1216-1239) (GenBank Accession No.: U83509)
Ang-2	S1: CTG ATG TGG AAG CCC AAG TAC TAA (nt. 638-661) S2: CAG AC(C/G) AC(A/G) AGA CT(C/T) GA(A/G) CT(T/G) CAG C (nt. 664-688) S3: TGT CCA ATG C(C/T)G TGC AGA GGG A (nt. 392-413) AS1: GTC ACA GAT GGC CTT GAT CTC (nt. 1132-1152) AS2: GTG TAG ATG CCA (C/T)T(T/G) GTG GTG (A/T)GT (nt. 1082-1106) AS3: CAC TGG TCT G(A/G)T CCA AAA TCT G (nt. 727-748) AS4: TCA TG(C/T) TGC TGC TTC TGA AG (nt. 940-959) (GenBank Accession No.: AF004326)
Ang-3	S: GTG TTC CAG GAC TGT GCA GA (nt. 877-896) AS: AAT TAG TGC CAG CGG ATG CC (nt. 1450-1469) (GenBank Accession No.: AF113707)
Tie 1	S: GTC AGA CCG GAT CCC CCA GAT CCT C (nt.1390-1414) AS: CCT GGG GCCGGT AGT GCA GGC GG (nt.1792-1814) (GenBank Accession No.: X80764)
Tie 2	S: CAA CAT AGG ATC AAG CAA CCC AGC C (nt. 2296-2320) AS: GTC CCT GTG ATC ATC TTT GGA GGC (nt. 2729-2752) (GenBank Accession No.: L06139)

Table 2.2 **Optimal primer annealing temperatures, optimal concentration of magnesium chloride and optimal PCR cycle number used for each specific reaction**

Target	Annealing temperature	$MgCl_2$ concentration	PCR cycle number
Ang-1 S1+AS3 S2+AS2	 60 °C 55 °C	 2.0 mM 1.5 mM	 25 cycles 25 cycles
Ang-2 S1+AS1 S2+AS2	 55 °C 58 °C	 2.0 mM 2.0 mM	 19 cycles 25 cycles
Ang-3	58 °C	2.0 mM	35 cycles
Tie 1	55 °C	2.0 mM	35 cycles
Tie 2	58 °C	2.5 mM	35 cycles

Table 2.3 Expected product sizes used for each specific reaction

Primers	Types of Ang-1 or Ang-2 isoforms detected	Expected product size for each Ang-1 isoform
Ang-1 (S1 + AS3)	Ang-1.5 kb	1013 bp
	Ang-1.3 kb	846 bp
	Ang-0.9 kb	374 bp
	Ang-0.7 kb	207 bp
Ang-1 (S2 + AS3)	Ang-1.5 kb	488 bp
	Ang-1.3 kb	321 bp
Ang-1 (S1 + AS2)	Ang-1.5 kb	893 bp
	Ang-0.9 kb	254 bp
Ang-2 (S2+AS2)	$Ang\text{-}2_{496}$	443 bp
Ang-2 (S3+AS3)	$Ang\text{-}2_{496}$	357 bp
	$Ang\text{-}2_{443}$	198 bp
Ang-2 (S3+AS4)	$Ang\text{-}2_{496}$	568 bp
	$Ang\text{-}2_{443}$	409 bp
Ang-3	-	593 bp
Tie 1	-	425 bp
Tie 2	-	457 bp


S1
S2
AS1

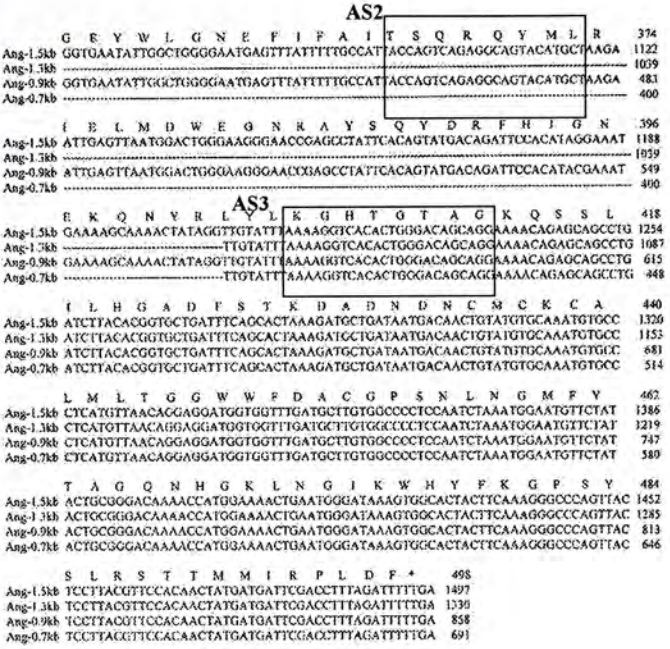
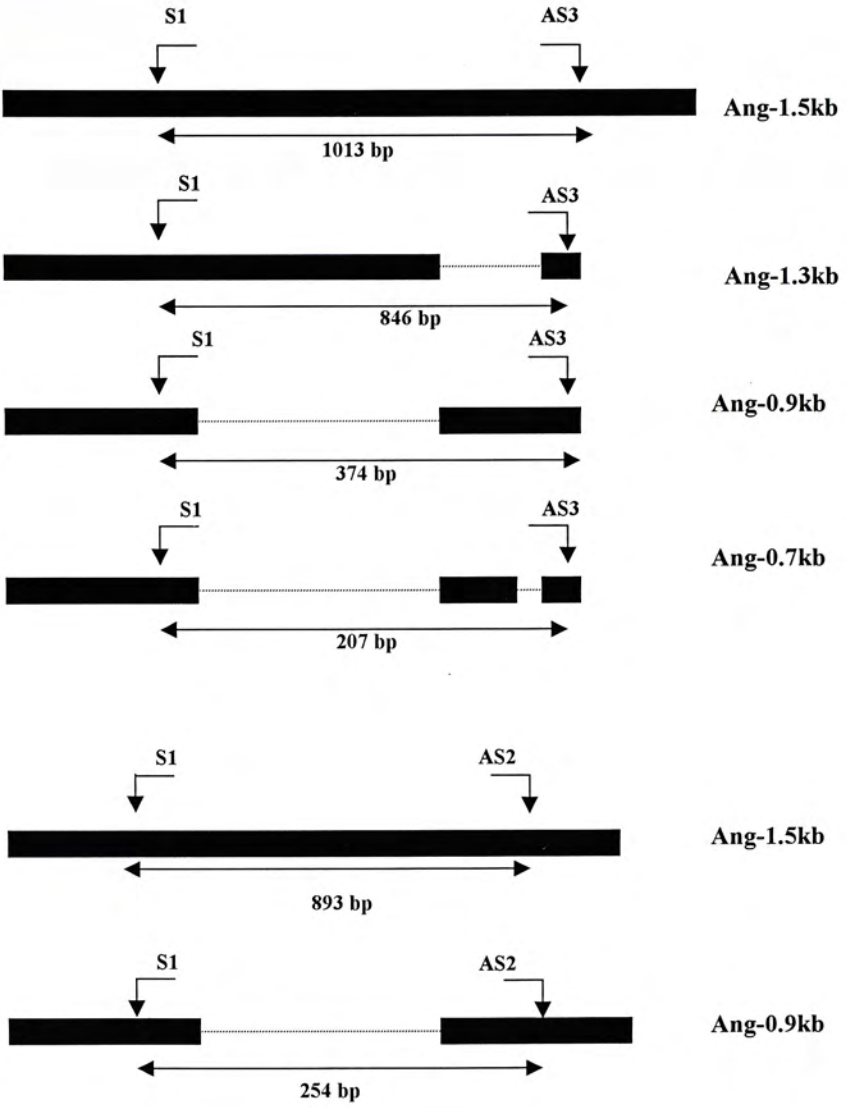


Figure 2.2 Positions of the primer sequences for the amplification of the four alternatively spliced species of Ang-1 from Huang *et al.* (2000). The positions of the primer sequences are boxed. The amino acid sequence of Ang-1.5 kb is shown above the nucleotide sequence. Dotted lines indicate the spliced sequences. Amino acid and nucleotide residues are numbered to the right of each sequence according to the mouse Ang-1 cDNA sequence submitted to GenBank with the following accession numbers of #U83509. The termination codon is indicated by an asterisk.


S1
AS3
Ang-1.5kb
1013 bp
S1
AS3
Ang-1.3kb
846 bp
S1
AS3
Ang-0.9kb
374 bp
S1
AS3
Ang-0.7kb
207 bp
S1
AS2
Ang-1.5kb
893 bp
S1
AS2
Ang-0.9kb
254 bp

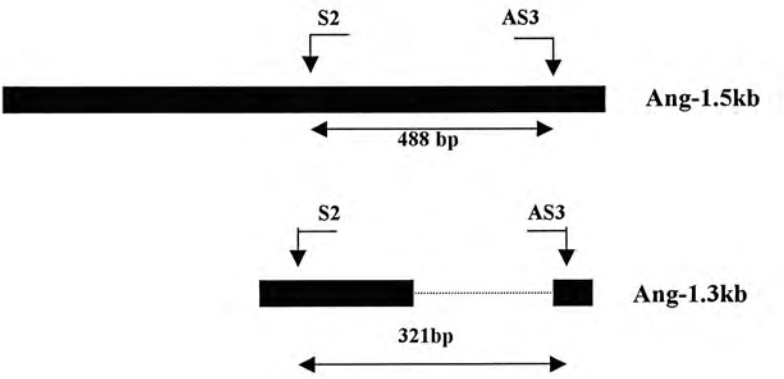


Figure 2.3 Schematic diagrams showing the generation of various lengths PCR products using a combination of primers, when in the presence of alternatively spliced variants of Ang-1 mRNA. Ang-1.5 kb encodes the full-length Ang-1. The thick solid lines represent fragments derived from various forms of Ang-1 and are not drawn to scale. The alternatively spliced parts of Ang-1 are shown as broken lines. The nucleotide sequence is quoted from the previous study [Huang *et al.*, 2000].

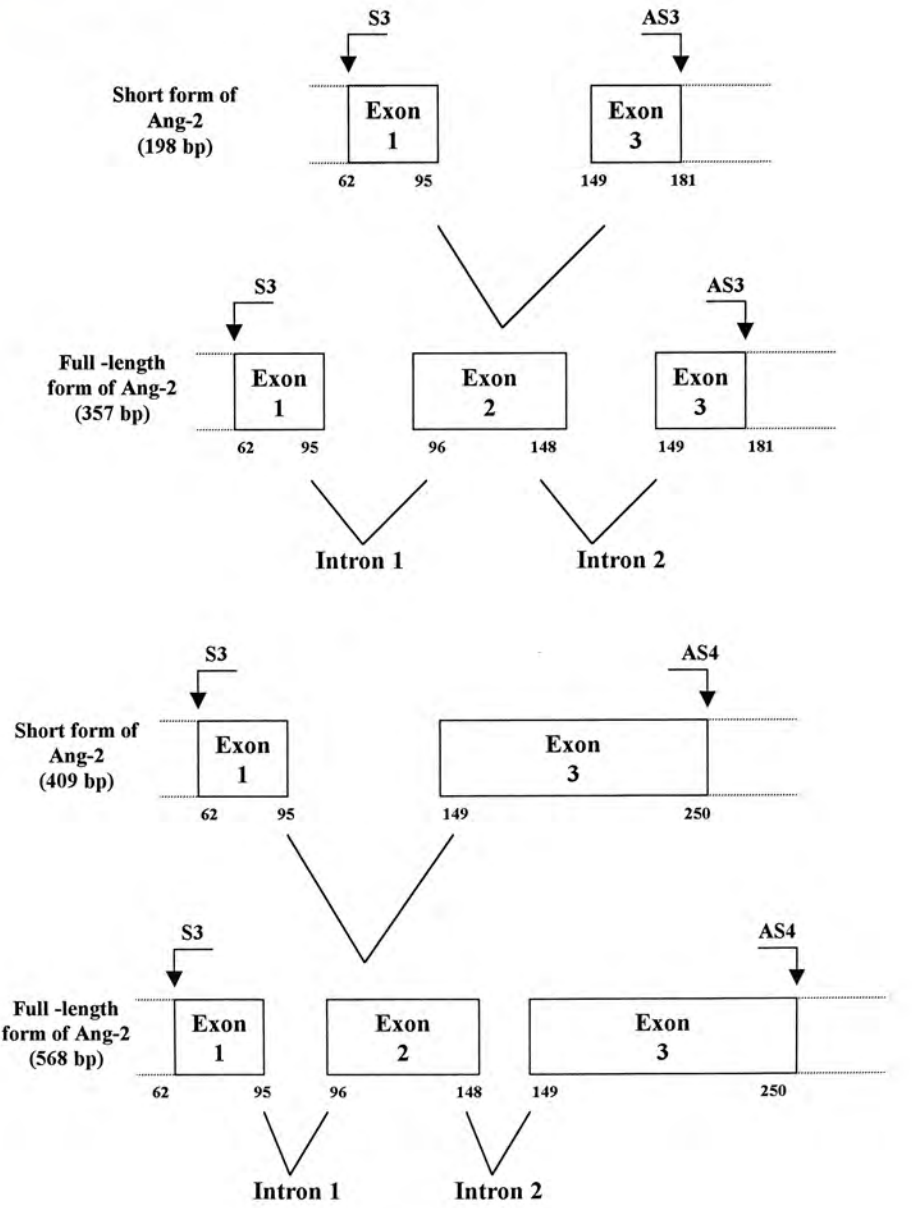


Figure 2.4 Genomic organization of the human Angiopoietin 2 gene. Exons are marked inside of boxes, *Exon 1*, *Exon 2* and *Exon 3*. Lower numbers denote amino acid numbers. Alternative splicing of *Exon 2* (amino acids 96-148) gives rise to the short form of human Ang-2.

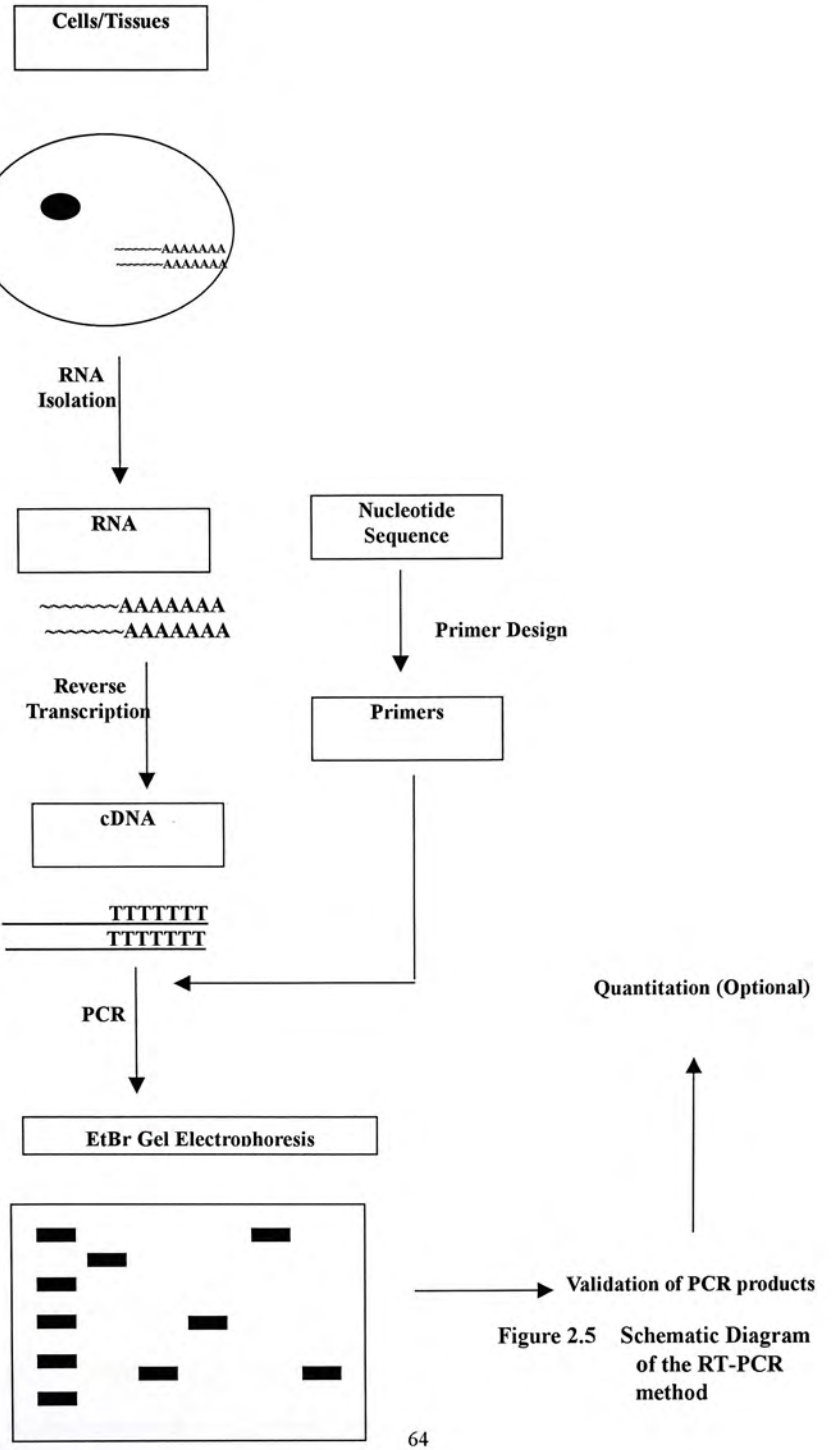


Figure 2.5 Schematic Diagram of the RT-PCR method

2.3.6 Purification of PCR products

The amplification products were purified using Gene-Clean Kit II (Bio 101). The specific bands identified on gel electrophoresis separation of PCR products were excised in a minimal volume of ethidium bromide-stained agarose gel with a razor blade under transillumination using long-wave ultraviolet light for as short a time as practical to avoid nicking of DNA. The agarose gel slices were placed inside a pre-weighed 1.5 mL microcentrifuge tube and the net weight of the gel was determined on an electronic balance. The approximate volume of the gel slices was estimated by equating 0.1 gram of gel to a volume of 100 μL. Three volumes of 6M sodium iodide were added to the agarose gel and incubated in a water bath at 55 ^{o}C with occasional mixing for 5 minutes or until the agarose had completely dissolved in the sodium iodide solution. 5 μL GLASSMILK suspension from the Gene-Clean Kit II (Bio 101) was then added to the tube, mixed and then incubated at room temperature for 5 minutes to allow the binding of the DNA. The GLASSMILK suspension is a specially prepared aqueous suspension of proprietary silica matrix that binds single and double stranded DNA but not other contaminants. Each sample was occasionally mixed for every 1-2 minutes to ensure that the GLASSMILK suspension remained suspended. At the end of the incubation, the GLASSMILK was separated by centrifugation at 11,600g for 5 seconds. The supernatant was discarded and the pellet was washed three times with 500 μL 'New Wash Solution'. This solution is a concentrated solution of sodium chloride, TRIS and EDTA to which water and ethanol are added. In each wash the pellet was completely resuspended by pipetting up and down a pipette tip and eventually the contents from two centrifuge tubes were pooled into one during the final wash. After the final centrifugation, as much 'New

Wash Solution' was removed from each tube as possible. The pellet was resuspended in 5 μL DEPC-treated water and then incubated in a water bath at 55 ^{o}C for 2-3 minutes twice. The tube was then centrifuged for 30 seconds at 11,600g and the supernatant was collected into a new 0.5 mL microcentrifuge tube. Resuspension and incubation of the pellet in DEPC-treated water were repeated one more time to recover DNA from the supernatant. One microlitre of the reconstituted PCR product was mixed with 8 μL of DEPC-treated water and 1 μL of bromophenol blue-xylene cyanol FF loading buffer, and loaded on to 1.2% ethidium bromide-stained agarose gel. A low DNA mass ladder (Gibco BRL, Grand Island, NY, USA) was also included to estimate the quantity of DNA recovered. The authenticity of the PCR product was verified, after purification from the PCR reaction, by either forward or reverse sequencing (or both).

2.3.7 Confirmation of PCR product authenticity by automated DNA sequencing

Sequencing was performed using a dRhodamine Terminator Cycle Sequencing Kit (Perkin Elmer, Applied Biosystems, California, USA) and an automated sequencer (Advanced ABI 310 Genetic Analyzer, Perkin Elmer, Tokyo, Japan). A 20-μL reaction mixture containing the following was subjected to the following cycling parameters: 26 cycles of 1 minute at 96 ^{o}C, 1 minute at 50 ^{o}C and 4 minutes at 60 ^{o}C. It included 30-50 ng PCR product purified using the Gene-Clean Kit II, 2 pmoles per μL of either sense or antisense primer, 8 μL Terminator Ready Reaction Mix (dRhodamine) (PE Bio Systems, California, USA) and DEPC-treated water. Cycling was performed on a [M. J. Research, Inc. PTC-

200] Thermal Cycler (PTC-200, M. J. Research, Inc., San Francisco, USA). The reaction product was then added to a 0.6 mL thin wall PCR tube (Robbins Scientific Corporation, San Aleso Avenue, Sunnyvale, CA) containing 2 μL 3 M sodium acetate and 50 μL 95% ethanol. The content was vortexed for a few seconds and then incubated at room temperature for 15 minutes to precipitate the extension products. Afterwards it was centrifuged at 11,600g for 20 minutes and the supernatant was immediately pipetted off with great care. The pellet which is often invisible was rinsed twice with 250 μL 70% ethanol and recovered after each wash by high speed centrifugation. Finally the pellet was dried under SpeedVac for 5-10 minutes and then reconstituted in 20 μL Template Suppression Buffer (TSR) (PE Applied Biosystems, USA). After further subjecting the sample to thorough mixing and incubation at 95°C for 2 minutes, it was quickly chilled on ice and loaded on to the automated DNA sequencer (Advanced ABI310 Genetic Analyzer, Perkin-Elmer, CA, USA). Sequencing data were submitted to the BLAST Search (NCBI, National Centre for Biotechnology Information; http://www.ncbi.nlm.nih.gov.BLAST) to confirm the authenticity of the nucleotide sequences of PCR products obtained from RT-PCR using different specific primer sets.

2.4 Western blot analysis

2.4.1 Preparation of cell lysates from primary testicular cells and testicular cell lines

Cytosolic protein was extracted from cell pellets of testicular cell lines and primary testicular cells. The cell pellet was washed three times with 10 mM phosphate-buffered saline (PBS, pH 7.4) to remove non-cellular proteins or other contaminants before resuspended in 4 volumes of lysis buffer (10 mM TRIS-HCl, pH 7.4) with the following protease inhibitors added (10 μg/μL leupeptin, 5 μg/μL aprotonin, 100 mM phenylmethylsulfonyl fluoride PMSF, 100 mM sodium orthovanadate, 200 mM EGTA and 200 mM EDTA). The cells were lysed by three repeated cycles of freezing and thawing which involved snap-freezing the cell pellet in lysis buffer in liquid nitrogen and followed immediately by thawing at room temperature. After that the cell lysates were cleared by centrifugation at 12,000 rpm for 15 minutes at 4 °C and the resulting supernatant was transferred to a new 1.5 mL microcentrifuge tube ready for protein determination.

2.4.2 Preparation of mouse testicular and adult rat testicular tissue lysates

Adult rat testes and mouse testes were decapsulated and had the testicular subcapsular artery removed before being homogenized in lysis buffer (with the same composition and protease inhibitors added as indicated in Section 2.4.1). Approximately 1 mL ice-cold lysis buffer was used for every 50-100 mg of testicular tissue being hand-homogenized using a 3 mL glass homogenizer with a clearance of 0.004-0.006 inch (Kontes Scientific Glasswarer/Instruments, Vineland, NJ, USA). The tissue homogenate was kept cold all the time by placing the homogenizer inside a bucket filled with crushed ice in water. At the end, the cell

lysates were transferred into 1.5 mL microcentrifuge tubes and cleared by centrifugation at 12,000 rpm for 15 minutes at 4 °C. The resulting supernatant was collected for protein determination.

Tissue lysate of the testicular subcapsular artery was prepared from pooled samples. They were homogenized in ice-cold RIPA buffer (1% NP40, 0.5% sodium deoxycholic acid, 0.1% SDS in 98mL PBS) supplemented with the protease inhibitors (10 μ g/ μ L leupeptin, 5 μ g/ μ L aprotonin, 100 mM phenylmethylsulfonyl fluoride PMSF, 100 mM sodium orthovanadate, 200 mM EGTA and 200 mM EDTA). The tissue homogenate was left on ice in the RIPA buffer for 30 minutes before cleared by centrifugation at 20,000g for 20 minutes at 4 °C. The resulting supernatant was collected for protein determination.

2.4.3 Determination of protein concentration

Protein determination was performed using the Bio-Rad DC Protein Assay (Bio-Rad, CA, USA) according to the manufacturer's instructions. A series of bovine serum albumin (BSA) standards of different concentrations was prepared using the same lysis buffer as the samples (0.1-2.0 mg/mL). The unknown samples were diluted to a ratio of 1/10, 1/20 or 1/50 and mixed well before being assayed. 5 μL of the above working standards, blank (the same lysis buffer for sample preparation) or diluted unknown samples were added to the wells of a 96-well ELISA plate (Nunclon) and assayed in duplicate. Working reagent A was prepared by mixing 1 mL reagent A with 20 μL Reagent S and 25 μL were added into each sample well and mixed gently by tapping and rocking. This was followed by the addition of 200 μL reagent B and the assay mixture was incubated at room

temperature for 15 minutes. At the end, absorbance in each well was read using an ELISA plate reader (Dynatech Laboratories HK Ltd). The protein concentrations of unknown samples were calculated based on the standard curve. Samples were then aliquoted in an appropriate volume that contained the desired amount of total protein (usually 150 μg) and the ampoules were stored frozen at –80°C until use for Western immunoblotting.

2.4.4 Reagents for Western blot analysis

A 10% sodium dodecyl-sulphate (SDS) solution was prepared by adding 10 g electrophoretic-grade reagent (#L3771; Sigma, St. Louis, MO, USA) to 100 mL deionized water.

A 4x stock TRIS-glycine electrophoresis buffer was prepared by first dissolving 30 g electrophoretic-grade TRIS base (Bio-Rad, CA, USA) and 144 g electrophoretic-grade glycine (Bio-Rad, CA, USA) in 2000 mL deionized water. The pH was then adjusted to 8.3 before the final volume was made up to 2.5 litres using deionized water. Working TRIS-glycine electrophoresis buffer was prepared just before use by mixing 175 mL 4x stock solution with 7 mL 10% SDS and making up to a final volume of 700 mL using deionized water.

A Transfer Buffer with SDS was prepared by dissolving 5.82 g TRIS base (Bio-Rad, CA, USA) and 2.93 g glycine (Bio-Rad, CA, USA) in deionized water, and after adding in 3.75 mL 10% SDS and 200 mL methanol, the final volume was made up to 1 litre using deionized water without any adjustment of pH.

A phosphate buffer saline tween-20 (PBST) solution was prepared by adding 80 g sodium chloride (BDH Chemicals Ltd, Poole England), 15.601 g hydrated sodium dihydrogen phosphate monohydrate (Merck, Darmstadt,

Germany) and 2 g potassium chloride (Merck, Darmstadt, Germany) to 2.5 litres deionized water. The pH was adjusted to 7.4-7.5 before 50 mL Tween-20 (polyoxyethylene sorbitan monolaurate) (#P1379, Sigma, St. Louis, MO, USA) was added and the final volume made up to 10 litres using deionized water.

A 30% acrylamide/bisacrylamide solution was prepared by dissolving 29.2 g electrophoretic-grade acrylamide (Bio-Rad, CA, USA) and 0.8 g bisacrylamide (N,N'-bis-methylene-acrylamide) in 100 mL deionized water. It was then filtered and stored in the dark at 4°C until use.

A 1.5 M Lower TRIS buffer was prepared by dissolving 18.17 g electrophoretic-grade TRIS base (Bio-Rad, CA, USA) and 4 mL 10% SDS in 60 mL deionized water. The pH was adjusted to 8.8 before the final volume was made up to 100 mL using deionized water. A 0.5M Upper TRIS buffer was prepared by adding 6.057 g electrophoretic-grade TRIS base (Bio-Rad, CA, USA) and 4 mL 10% SDS to 70 mL deionized water. The pH was adjusted to 6.8 before the final volume was made up to 100 mL with deionized water.

All reagents for Western blot analysis were stored at 4 °C before use.

2.4.5 Preparation of protein samples and markers for Western blot analysis

One volume of stock β-mercaptoethanol (Amresco) was added to 9 volumes of 2x electrophoresis sample buffer (125 mM TRIS-HCl, pH 6.8; 4% 10% SDS; 20% glycerol and 0.06% bromophenol blue) just before use. The protein samples were diluted using 10 mM TRIS-HCl (pH 7.4) to give 150 μg total protein in 25 μL and this was then added to an equal volumes of 2x electrophoresis sample buffer (with β-mercaptoethanol). As a result, 20 μL of each diluted protein

sample (in electrophoresis sample buffer) contained 60 μg total protein. The protein samples, together with the biotinylated and prestained markers used in the gel electrophoresis, were denatured by boiling in a water bath at 100°C for 5 minutes before 20 μL were loaded on to the gel. The biotinylated marker 6B (#B8146, Sigma, St. Louis, MO, USA), or 7B (#B2787, Sigma, St. Louis, MO, USA) was diluted 1/20 by mixing 5 μL stock with 45 μL 10 mM TRIS-HCl (pH 7.4) followed by the addition of 50 μL 2x electrophoresis sample buffer (with β-mercaptoethanol). The prestained markers - M2 (#G6017, M.W. 116 kDa) and M3 (#G6017, M.W. 35.2 kDa) (Sigma, St. Louis, MO, USA), were similarly prepared.

2.4.6 Sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The Mini-PROTEAN II Electrophoresis Cell Assembly (Bio-Rad Laboratories, Hercules, California, USA) was used to prepare 10% SDS-PAGE of 0.75 mm thick and 60 mm (H) x 85 mm (W) in area. Sixty μg of proteins in 20 μL were added to each sample well.

Ten percent (10%) lower separating gel layer was prepared by mixing 3.34 mL deionized water, 2.0 mL 1.5 M Lower TRIS buffer (pH 8.8) and 2.66 mL 30% acrylamide/bisacrylamide solution in a glass vial. The mixture was degassed under vacuum for 20 minutes since oxygen in the solution could inhibit the subsequent polymerization step. Also, the presence of gas would lead to the formation of gas bubbles in the gel during the process of electrophoresis. 10% ammonium persulphate (APS) solution (Amersham Pharmacia Biotechnology, Buckinghamshire, UK) was freshly prepared by adding 0.05 g APS to 500 μL

deionized water in a 1.5 mL microcentrifuge tube. 8 µL N,N,N,-N'-tetramethylethylene-diamine (TEMED, Bio-Rad, CA, USA) and 80 µL freshly prepared 10% APS were then added to the degassed acrylamide/bisacrylamide solution to initiate the polymerization reaction. 3.7 mL of this separating gel solution was immediately poured into the space between the two glass plates of the gel casting cassette. Care was taken to minimize the mixing of this solution with air. 200 µL isopropyl alcohol was carefully layered on the top of this gel layer to prevent its contact with air and to ensure its interface with the stacking gel is leveled. The gel was left to polymerize for 40 minutes. Afterwards the overlay isopropyl alcohol was poured off and deionized water was used to completely rinse off any residues that remained. The last drop of water above the separating gel was removed by absorption with filter paper without damaging the surface of the gel.

Four percent (4%) stacking gel mixture was prepared by mixing 1.9 mL deionized water, 0.75 mL 0.5M Upper TRIS buffer (pH 6.8), 0.4 mL 30% acrylamide/bisacrylamide, 6 µL of TEMED and 28 µL of APS. One mL of this solution was layered on top of the separating gel until overflow. The sample comb was then inserted and placed centrally in a levelled position. The stacking gel was allowed to polymerize for 40 minutes. The sample comb was then carefully removed and the wells were thoroughly rinsed with Running Buffer before they were completely filled using the same buffer.

The heat denatured protein samples and markers were loaded individually into each sample well cast in the stacking gel. And after filling the upper and lower buffer chamber with the Running Buffer, electrophoretic separation of the proteins was carried out at 135 volts (PowerPac 200 Power Supply, Bio-Rad Laboratories,

Hercules, California, USA) until the prestained molecular weight marker had migrated to the desired position.

2.4.7 Transfer of proteins to membrane

After gel electrophoresis was completed, the gel was carefully removed from the electrophoretic unit and placed face up. The upper portion representing the stacking gel was cut away.

Polyvinylidene difluoride membrane (PVDF, Immobilon-P^{SQ}, Millipore Corporation, Bedford, USA) cut to a size of 9 cm x 5 cm was wetted with methanol for 15 seconds and then thoroughly rinsed with transfer buffer before use. The SDS-PAGE gel was also allowed to equilibrate in the transfer buffer for 20 minutes with mixing over a Gyro Rocker (STR9, Stuart Scientific, Staffordshire, UK) before subjected to electro-blotting.

Electro-blotting was carried out using a Trans-Blot Semi-Dry Electrophoretic Transfer Cell (#170-3940, Bio-Rad Laboratories, Hercules, California, USA), following the supplier's instruction. The gel was placed on top of a PVDF membrane and then sandwiched between sheets of Extra Thick Blot Absorbent Filter Paper (#170-3960, Bio-Rad Laboratories, Hercules, California, USA) (one sheet underneath and two sheet above) pre-soaked in transfer buffer. The stack was placed between the electrodes of the semi-dry transfer cell and electroblotting was allowed to proceed at 19V for 50 minutes.

2.4.8 Blocking of the membrane

After transfer, the membrane was briefly rinsed three times with deionized water. Blocking of non-specific binding sites on the blot was achieved

by immersing the blot in 1% gelatin-PBST (0.6 g gelatin and 60 mL PBST) (#G9391, Sigma, St. Louis, MO, USA) with rotary mixing inside a hybridization oven (Biometra, Germany) maintained at 25°C for 45 minutes. After that the membrane was briefly rinsed in three changes of PBST (10 minutes each) at room temperature to remove the blocking solution.

2.4.9 Immunoblotting

For the immunoblotting of Ang-1, three commercially available antibodies (Santa Cruz Biotechnology, Inc. Santa Cruz, California) had been tested. One is a rabbit polyclonal antibody [Ang-1 (H-98), #sc-8357] raised against the amino acids 400-498 mapping at the carboxyl terminus of Ang-1 of human origin. The other two are goat polyclonal antibodies mapping to epitope at the carboxy terminus of human Ang-1 [Ang-1 (C-19), #sc-6320] and amino terminus of human Ang-1 [Ang-1 (N-18), #sc-6319], respectively. Based on information provided by the supplier, Ang-1 (H-98) exhibits cross-reactivity with Ang-2, while the other two antibodies are Ang-1 specific. For the immunoblotting of Ang-2, only one commercially available antibody (Santa Cruz Biotechnology, Inc. Santa Cruz, California) had been tested. This antibody is a goat polyclonal antibody mapping to epitope at the carboxy terminus of human Ang-2 [Ang-2 (C-19), #sc-7015]. These primary antibodies were used at a dilution of 1:500 in PBST and incubated with the blot overnight at room temperature on a Roller Mixer (SR1, Stuart Scientific, Staffordshire, UK).

After incubation with the primary antibody, the blot was washed three times (20 minutes each) with PBST before exposing to horse radish peroxidase (HRP)-conjugated protein-A or HRP-conjugated secondary antibody. This allowed the

detection and amplification of signals originating from primary antibody binding to specific protein bands on the blot. For rabbit polyclonal primary antibody, protein A-conjugated HRP (Santa Cruz Biotechnology, Inc.) was used at 1:5000 dilution while for goat polyclonal primary antibody, rabbit anti-goat IgG-conjugated HRP (Zymed Laboratories, South San Francisco, California) was used at 1:2500 dilution. They were incubated with the blot for 30 minutes at room temperature.

Finally after further rinsing with PBST, the antibody binding was visualized using ECL Western blotting detection reagent (Amersham Pharmacia Biotechnology, Buckinghamshire, UK). The blot was incubated with the ECL reagent for 1 minute at room temperature without agitation. The excess reagent was drained off and the blot was wrapped in GladWrap. The pattern of chemiluminescence on the blot was recorded by exposing it to an X-ray (Biomax MS scientific imaging film, Kodak Eastman, Rochester, NY, USA) at room temperature inside a film cassette for a duration dependent on the intensity of the positive signals. The position of biotinylated protein molecular size markers was subsequently determined through another incubation of the blot with streptavidin-conjugated HRP (1:5000 dilution, Zymed Laboratories) at room temperature for 30 minutes, and then followed by the exposure to ECL reagents and then the X-ray film.

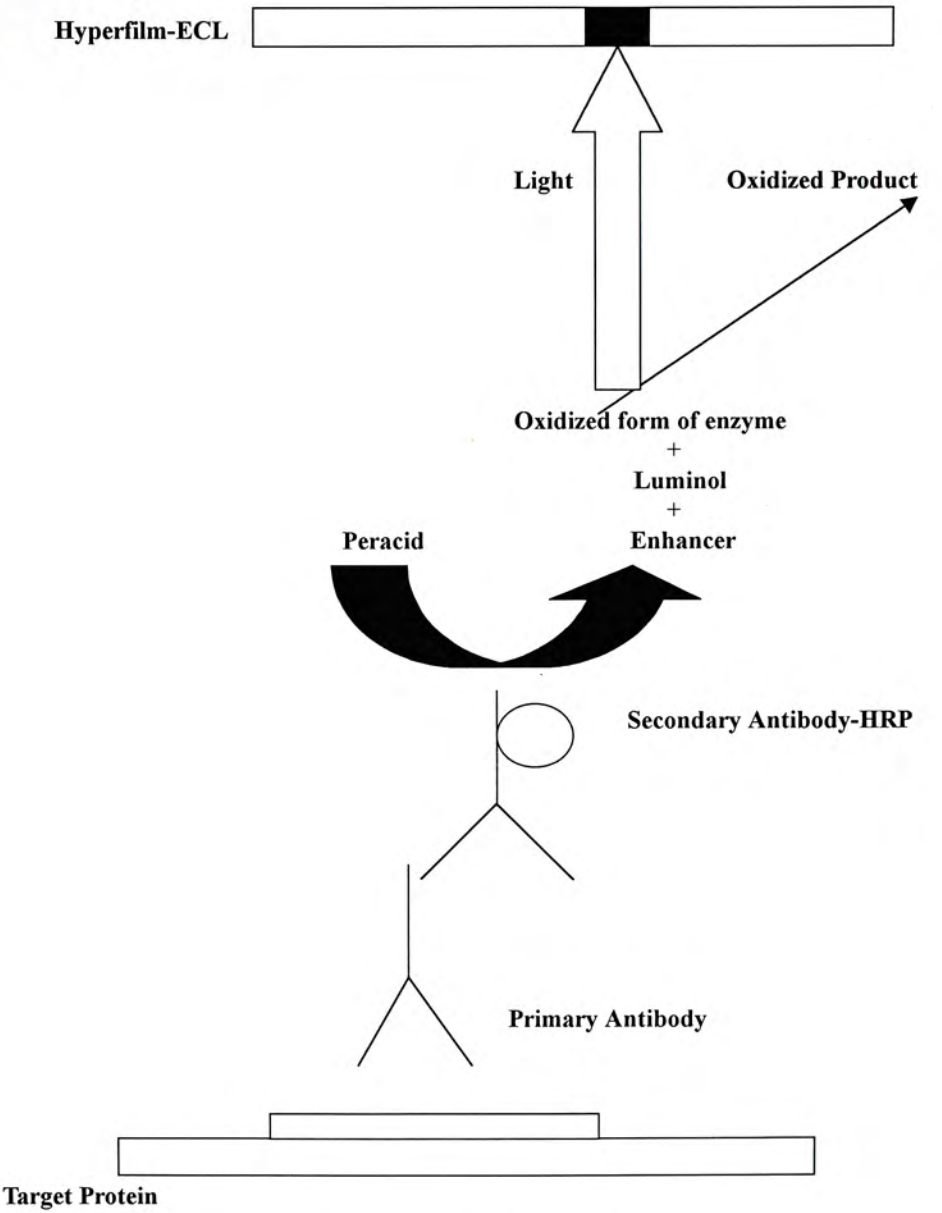


Figure 2.6 **Principles of Western blot analysis**

2.5 Immunohistochemical staining for Ang-1, Ang-2, Ang-3, Tie 1 and Tie 2 in rat testes

Ang-1, Ang-2, Ang-3, Tie 1 and Tie 2 were localized in rat testis sections using affinity-purified goat polyclonal [Ang-1 (N-18), #sc-6319], [Ang-2 (C-19), #sc-7015] and [Ang-1/4 (C-19), #sc-9360] antibodies and affinity-purified rabbit polyclonal [Tie 1 (C-18), #sc-342] and [Tie 2 (C-20), #sc-324] antibodies that are commercially available from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The [Ang-1 (N-18), #sc-6319] and [Ang-2 (C-19), #sc-7015] antibodies are raised against a peptide mapping at the N-terminus of Ang-1 of human origin and C-terminus of Ang-2 of human origin, respectively. [Ang-1/4 (C-19), #sc-9360] antibody is raised against a peptide mapping at the C-terminus of Ang-4 of human origin. Tie 1 [(C-18), #sc-342] and Tie 2 [(C-20), #sc-324] antibodies are raised against the first 20 amino acids in the C-terminus of Tie 1 of human origin and Tie 2 of mouse origin, respectively. Based on information provided by the supplier, all these antibodies cross-react with the corresponding antigens in mouse, rat and human. [Ang-1/4 (C-19), #sc-9360] antibody exhibits cross-reactivity with both Ang-1 and Ang-3 of mouse and rat origin, and Ang-1 and Ang-4 of human origin.

The testes were collected from adult Sprague-Dawley rats and individually placed inside a mould made out of aluminum foil and filled with the embedding medium (OCT compound; Miles Scientific, Elkhart, IN). They were then rapidly frozen in liquid nitrogen to create a more uniform block to facilitate sectioning. The testes once embedded were kept frozen at -80 ^{o}C until use for cryostat sectioning. Six microns sections were prepared on a cryotome (Shandon AS620E, Cryotome, Cheshire, UK) and mounted on glass slides (Sail Brand,

China) coated with 1% 3-aminopropyltriethoxysilane (APES) (Sigma, St, Louis, MO, USA) in ethanol. For [Ang-1 (N-18), #sc-6319], [Ang-1/4 (C-19), #sc-9360], [Tie 1 (C-18), #sc-342] and [Tie 2 (C-20), #sc-324] antibodies, the sections were lightly fixed in freshly prepared 4% paraformaldehyde at room temperature. For [Ang-2 (C-19), #sc-7015] antibody, the sections were fixed in cold acetone at -20 ^{O}C for 10 minutes. After that, they were processed for streptavidin-biotin-peroxidase complex immunostaining. The acetone-fixed sections had to be air-dried completely at room temperature before proceeding with staining. The sections were equilibrated in three washes (2 minutes each) of 10 mM phosphate-buffered saline (PBS, pH 7.4) and the endogenous peroxidase activity was quenched in 0.6% freshly prepared hydrogen peroxide solution in PBS for 20 minutes. This was followed by blocking with neat fetal bovine serum (FBS) for 15 minutes at room temperature to reduce the non-specific binding. At the end of incubation, the FBS was simply poured off and the primary antibody added. The sections were incubated with a 1:100 dilution of primary antibody in a solution of 20% fetal bovine serum in PBS for 1 hour at room temperature for [Tie 1 (C-18), #sc-342], [Tie 2(C-20), #sc-324] and [Ang-1/4 (C-19), #sc-9360] antibodies or 1 hour at 37 ^{O}C for [Ang-1 (N-18), #sc-6319] and [Ang-2 (C-19), #sc-7015] antibodies in a moist chamber. After the primary antibody incubation, the sections were rinsed with three washes (2 minutes each) of PBS before the corresponding biotinylated secondary antibody was added and incubated for 10 minutes at room temperature. For [Ang-1 (N-18), #sc-6319], [Ang-2 (C-19), #sc-7015] and [Ang-1/4 (C-19), #sc-9360] antibodies, biotin-rabbit anti-goat secondary antibody was used, whereas for [Tie 1 (C-18), #sc-342] and [Tie 2 (C-20), #sc-324], biotin-goat anti-rabbit secondary antibody was used. Both

secondary antibodies were from Zymed Laboratories (South San Francisco, California, USA) and used at 1:75 dilution in PBS. After further washes (3 times, 2 minutes each) with PBS, streptavidin-conjugated horse radish peroxidase (Zymed Laboratories) diluted 1:400 in PBS was added to the sections and incubated for 10 minutes at room temperature. This was followed by further washings with PBS (3 times, 2 minutes each) and incubation at room temperature with the chromogen - 3-amino-9-ethylcarbazole (AEC) (Zymed Laboratories), for the localization of specific primary antibody binding to the testis sections. The duration of incubation with chromogen depended on the intensity of the colour development on the testis section. This was determined by regular checking under light microscope and terminated by washing with deionized water. The sections were then mounted in aqueous mountant consisting of 1 g/mL polyvinyl pyrrolidone, 1 mL glycerol, a small crystal of thymol in 25 mL deionized water, with or without prior counterstaining with haematoxylin, and covered with glass cover slips (22 x 22mm or 22 x 40 mm; Deckgläser, Germany). The sections were examined under light microscope (Optiphot-2, Nikon, Japan) and areas of specific staining were recorded using the Nikon Microflex photographic system.

3. Results

3.1 Expression of Ang-1 and Ang-1 alternatively spliced transcripts in the testis and other testicular cell types

3.1.1 Detection of Ang-1 expression in the testis and testicular cell types by nested PCR

In the preliminary studies using the RT-PCR approach, it was found that Ang-1 mRNA expression was weak in some testicular cell lines or increasing the cycle number resulted in multiple non-specific products. This could be explained by the low gene copy number of mRNA transcripts and the interference caused by contaminants or RNA degradation commonly found in nucleic acid preparations. Therefore, nested PCR was performed to increase the specificity and sensitivity of the mRNA detection (as shown in Figure 3.2).

The product from the first stage of the PCR reaction was diluted to an overall ratio of 1/1000 for the subsequent second stage of the PCR reaction. A housekeeping gene - β-actin, was used as an internal control to adjust the amount of template used in each PCR reaction, while C6 rat glioma cells served as a positive control(s) for the gene expressions of Ang-1 [Holash *et al.*, 1999a] and Ang-2 [Mandriota & Pepper, 1998] analysed. Despite the use of equal volumes of RT products for the first stage of the PCR reaction, there was some variability in the expression levels of β-actin, which was used as an internal control, probably due to

differences in the efficiency of the reverse transcription reaction and inaccuracy in the quantitation of total RNA (Figure 3.1). In this respect, the use of β-actin as an internal control could avoid the problem of additional variation in PCR efficiency with large differences in the amount of template used.

Using the first pair of PCR primers [Ang-1 (S1+AS3)], a product of 1013 bp resulted. Then using a second pair of internal primers [Ang-1 (S2+AS2)] a product of 368 bp was formed. As shown in Figure 3.1, agarose gel analysis of ethidium bromide stained PCR products showed only a single band corresponding to the expected size of 368 bp. This was found to be present in total testicular tissue, primary Leydig cells, primary Sertoli cells, primary germ cells, rat testicular blood vessel, mouse testis as well as the positive control - C6 rat glioma cells.

3.1.2 Detection of Ang-1 expression in testicular cell lines by nested PCR

Based on the experiment using testicular tissues and primary cells, the results for the expression of Ang-1 in testicular cell types obtained from Section 3.1.1 were not conclusive since they were not pure cell populations and were susceptible to cross-contamination from other cell types. Thus the cell lines provide us with an alternative tool to determine whether it would be the Leydig cells, Sertoli cells or both that express Ang-1. Total RNA was prepared from TM3

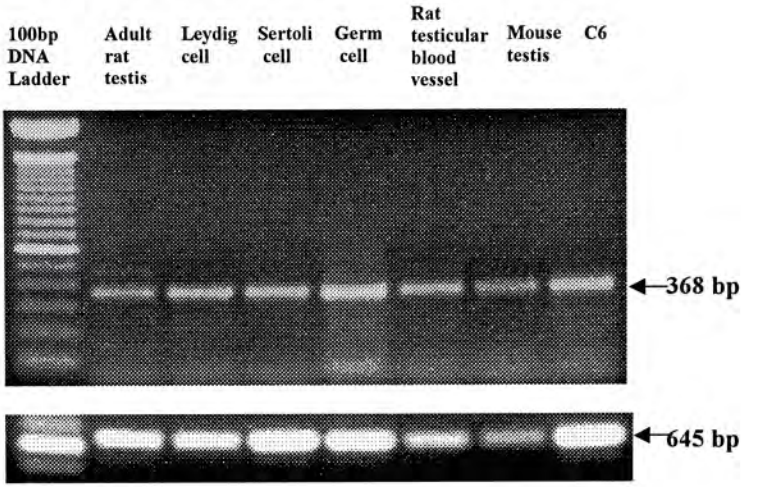


Figure 3.1 Analysis of Ang-1 expression in the adult rat testis and primary rat testicular cells using nested PCR. Products from the second stage of the nested PCR were separated in a 1.2% ethidium bromide-stained DNA agarose gel. The positive bands show the product of interest, which correspond to the expected size of 368 bp for Ang-1. The lower panel shows the corresponding signal intensity for β-actin using the same amount of template.

mouse Leydig cell, TM4 mouse Sertoli cell, LC540 rat Leydig tumour cells, MLTC-1 mouse Leydig tumour cells and R2C rat Leydig tumour cells. Similar nested PCR approach was used employing the same sets of external primers and internal primers for generating the results shown in Section 3.1.1. The product from the first stage of the PCR reaction was diluted to an overall ratio of 1/1000 (but 1/500 for MLTC-1 cells) for the subsequent second stage of the PCR reaction.

The weak expression of β-actin in MLTC-1 and LC540 testicular cells appeared to be resulted from an overestimation of RNA concentrations. Nevertheless, LC540 testicular cells could still strongly express Ang-1.

After nested PCR amplification, a 368-bp band of expected size was amplified and observed (as shown in Figure 3.2). Among these testicular cell lines, TM4, LC540 and R2C testicular cells were shown to express Ang-1 mRNA. On the contrary, the expression of Ang-1 was almost undetectable in TM3 and MLTC-1 Leydig cells. Although the signal for β-actin was low for LC540 rat Leydig tumour cells, suggesting that the amount of template used was low, Ang-1 transcripts could still be detected in this cell line using RT-PCR.

3.1.3 Sequence analysis of Ang-1 transcript amplified from adult rat testis

At the start of the study, the presence of Ang-1 transcripts in the rat testis

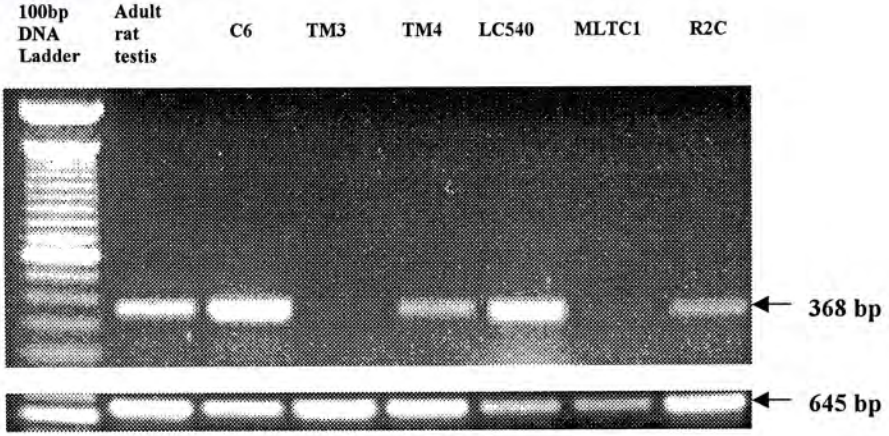


Figure 3.2 Analysis of Ang-1 expression in testicular cell lines using nested PCR. Products from the second stage of the PCR reaction were separated in a 1.2% ethidium bromide-stained DNA agarose gel. The positive bands correspond to the product of interest having the expected size of 368 bp for Ang-1. The lower panel shows the corresponding signal intensity for β-actin using the same amount of template.

was confirmed by sequencing the product from PCR reaction using a pair of primers which gave reasonably strong signal for the rat testis but not for the other testicular cell lines. However, the primer sets used in this part are different from those in the first part of the study so that it was unable to confirm the identity of the nested PCR products shown in Figure 3.1 and Figure 3.2 (Sections 3.1.1 and 3.1.2). For PCR amplification of Ang-1 using cDNA prepared from adult rat testis as template, the upstream primer sequence was 5'-CCGGATTTCTCTTCCCAGAAAC-3'(S1), corresponding to nt. 227-248, whereas the downstream primer sequence was 5'-CCAGCTTGATATACATCTGCACAGTCTC-3'(AS1), corresponding to nt. 1157-1184 (GenBank Accession Number – mouse Ang-1: U83509). These primers were designed based on the consensus regions of the mouse (GenBank Accession Number: U83509) and human (GenBank Accession Number: U83508) Ang-1 cDNA sequences. With this primer pair, Ang-1 mRNA was readily detected in adult rat testis without the need for nested PCR, and the product was targetted for the cDNA sequencing. The concentration of magnesium chloride used in the PCR reactions was 1.5 mM. The PCR parameters were denaturation at 94 °C for 1 minute, primer annealing at 61 °C for 45 seconds and extension at 72 °C for 1 minute 30 seconds for 40 cycles. Using this set of primers, it gives a product with

the expected size of 626 bp.

By searching Standard Nucleotide-nucleotide BLAST Database as described under Materials and Methods (Section 2.3.7), the nucleotide sequence of the PCR products obtained from adult rat testis was found to agree well with the known partial nucleotide sequence of rat Ang-1 (96.7% identity) (as shown in Figure 3.3). Therefore, the PCR products obtained using this pair of PCR primers [Ang-1 (S1+AS1)] could be proved to be the authentic fragments of Ang-1.

3.1.4 Detection of alternatively spliced species of Ang-1 mRNA in the testis and other testicular cell lines

In the first series of experiments, Ang-1 expression was investigated in the testis, primary testicular cells and several testicular cell lines using nested RT-PCR approach. The full-length Ang-1.5kb is encoded by a single-copy gene, that engenders multiple transcripts coding for the alternatively spliced transcripts of Ang-1, Ang-1.5 kb, Ang-1.3 kb, Ang-0.7 kb and Ang-0.9 kb [Huang *et al.*, 2000].

RT-PCR was performed to analyze the expression of the four alternatively spliced species of Ang-1 mRNA with Ang-1 (S1+AS2), Ang-1 (S2+AS3) and Ang-1 (S1+AS3) primer pairs, and if so to determine whether specific differences exist in the mRNA isoform(s) in adult rat testis when compared

```
mAng-1  481    acagagcagt acaacaccaa cgctctgcaa agggatgctc cacacgtgga gccggatttc
r-testis
rAng-1
hAng-1         acagaccagt acaacacaaa cgctctgcag agagatgctc cacacgtgga accggatttc


mAng-1  541    tcttcccaga aacttcagca tctggagcat gtgatggaaa attatactca gtggctgcaa
                         |||| |||||||||| |||||||||| |||||||||| ||||||||||
r-testis                 agca tctggagcat gtgatggaaa attatactca gtggctgcaa
                                                   |||| |||||||||| ||||||| ||
rAng-1                                             gaaa attatactca gtggctggaa
hAng-1         tcttcccaga aacttcaaca tctggaacat gtgatggaaa attatactca gtggctgcaa


mAng-1  601    aaacttgaga attacattgt ggaaaatatg aagtcggaga tggcccagat acaacagaat
               |||||||||| |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
r-testis       aaacttgaga attacattgt ggaaaatatg aagtcggaga tggcccagat acaacagaat
               |||||||||| |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
rAng-1         aaacttgaga attacattgt ggaaaatatg aagtcggaga tggcccagat acaacagaat
hAng-1         aaacttgaga attacattgt ggaaaacatg aagtcggaga tggcccagat acagcagaat


mAng-1  661    gctgttcaaa accacacggc caccatgctt gagataggaa ccagtctctt atctcagact
               |||||||||| |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
r-testis       gcggttcaaa accacacggc caccatgctg gagataggaa ccagcctctt gtctcagact
               |||||||||| |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
rAng-1         gcggttcaaa accacacggc caccatgctg gagataggaa ccagcctctt gtctcagact
hAng-1         gcagttcaga accacacggc taccatgctg gagataggaa ccagcctcct ctctcagact


mAng-1  721    gcagagcaga cccgaaagct gacagatgtt gagacccagg tactaaatca aacatcccga
               |||||||||| |||||||||  ||||||||  |||||||||| |||||||||| |||||||||
r-testis       gcagagcaga cccgaaagct cacagatgtg gagacccagg tactaaatca aacatcccgt
               |||||||||| |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
rAng-1         gcagagcaga cccgaaagct cacagatgtg gagacccagg tactaaatca aacatcccgt
hAng-1         gcagagcaga ccagaaagct gacagatgtt gagacccagg tactaaatca aacttctcga


mAng-1  781    cttgaaatac aactgctaga gaattcatta tcaacataca agctagagaa gcaacttctc
               |||||||| | ||||||| |    | |||||| |||||||||| |||||||||| || ||||||
r-testis       cttgaaatcc aactgctggg agaatcatta tcaacataca agctagagaa aca-cttctc
               |||||||||| |||||||||    | |||||| |||||||||| ||||||| || ||| ||||||
rAng-1         cttgaaatcc aactgctgga gaattcatta tcaacataca agctagaaaa acagcttctc
hAng-1         cttgagatac agctgctgga gaattcatta tccacctaca agctagagaa gcaacttctt


mAng-1  841    caacagacaa atgaaattct gaagattcac gaaaaaaaca gtttactaga gcacaaaatc
               |||||||||| ||  |||||| |||||||||  | |||||||| ||||| |||| ||| ||||||
r-testis       caacagacaa attgaattct gaagattcag ggaaaaaaca gtttattaga gcataaaatc
               |||||||||| ||  |||||| ||||||||||   |||||||| |||||||||| ||||||||||
rAng-1         caacagacaa atgaaattct gaagattcag aaaaaaaaca gtttattaga gcataaaatc
hAng-1         caacagacaa atgaaatctt gaagatccat gaaaaaaaca gtttattaga acataaaatc


mAng-1  901    ttagaaatgg agggaaaaca caaagaagaa ttggacacct tgaaggagga gaaagaaaac
                |||||||||| |||| |||   ||| |||||   ||| ||||   |||||||||| ||| ||||||
r-testis       ctagaaatgg aggggaaaac caaggaagag gtggncaccn ngaaggagga gaaggaaaac
               ||||||
rAng-1         ctagaa
hAng-1         ttagaaatgg aaggaaaaca caaggaagag ttggacacct taaaggaaga gaaagagaac


mAng-1  961    cttcaaggct tggtttctcg tcagacattc atcatccagg agttggagaa gcaacttagt
                | |||||   ||||| |||| |||||||||| |||||||| | |  ||| ||| ||||| |||
r-testis       atccaaggnn tggttactcg tcagacattc atcatccaag aagtggngaa gcaacntagc
rAng-1
hAng-1         cttcaaggct tggttactcg tcaaacatat ataatccagg agctggaaaa gcaattaaac
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mAng-1 1021    agagctacca acaacaacag catcctgcag aagcaacaac tggagctcat ggacacagtt
               |||| ||||| |||||||||| | |||||| |||||||||| ||| | ||| ||||||||
r-testis       agaggtacca acaacaacag tgttctgcag aagcaacaac tggggtgcat ggacacagcc
rAng-1
hAng-1         agagctacca ccaacaacag tgtccttcag aagcagcaac tggagctgat ggacacagtc


mAng-1 1081    cataaccttg tcagcctttg cactaaagaa ggtgttttgc taaagggagg aaaaagagaa
               ||||||| | ||||||| || ||| |||||| |||||||||| |||||||||| ||||||||||
r-testis       cataaccnng tcagcctntg cacaaaagaa ggtgttttgc taaagggagg aaaaagagaa
rAng-1
hAng-1         cacaaccttg tcaatctttg cactaaagaa ggtgttttac taaagggagg aaaaagagga


mAng-1 1141    gaagagaaac catttc***gaga ctgtgcagat gtatatcaag ctgg***tttaa taaaagtgga
               |||||||||| |||||
r-testis       gaagagaaac cattt
rAng-1
hAng-1         ggaagagaaa catttc***gaga ctgtgcagat gtatatcaag ctgg***ttta ataaaagtgga
```

Figure 3.3 Comparing the nucleotide sequence of the PCR product obtained using specific Ang-1 primers and cDNA from adult rat testis as template, against known partial sequence of rat Ang-1 and the corresponding regions of mouse and human Ang-1 cDNA sequence. mAng-1, rAng-1 and hAng-1 represent the published sequence of mouse, rat and human Ang-1, respectively (GenBank Accession Numbers - mAng-1: U83509, rAng-1: AF030376, and hAng-1: U83508). r-testis represents the nucleotide sequence of the PCR product amplified from adult rat testis using Ang-1 (S1+AS1) primers. The primer sequences are shown in italics and boldface. The positions of those nucleotide bases from r-testis that show exact match with the corresponding published sequence in the mouse and rat sequence, are indicated by short vertical bars. The numbering of nucleotide bases for mAng-1 is shown to the left of the sequence.

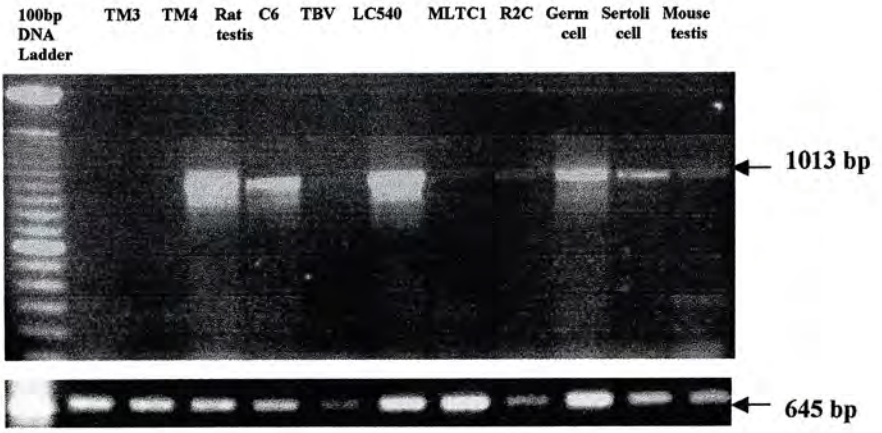
with human.

To confirm the presence of these transcripts in testis, new sets of primers were designed with the 5'end straddling the apparent splice junction site and the positions of the primer sequences were shown in Figures 2.2 and 2.3. Ang-1 (S1+AS3) primer pair was used for the detection of four alternatively spliced transcripts of Ang-1 (Ang-1.5 kb, Ang-1.3 kb, Ang-0.9 kb and Ang-0.7 kb) to give the predicted 1013-bp band, 846-bp band, 374-bp band and 207-bp band respectively. Ang-1 (S2+AS3) primer pair was used to detect the 1.5-kb and 1.3-kb isoforms and designed to give the predicted 488-bp band and 321-bp band respectively. Ang-1 (S1+AS2) primer pair was used to detect the 1.5-kb and 0.9-kb isoforms and designed to give the predicted 893-bp band and 254-bp band respectively.

Ang-1 (S1+AS3) primer pair was initially used for analyzing the expression of multiple isoforms of Ang-1 in primary testicular cells and testicular cell lines using RT-PCR, as it could detect all of the four alternatively spliced mRNA species of Ang-1. Since these isoforms were readily detectable in several human tumour cell lines [Huang *et al.*, 2000], therefore it would be interesting to determine whether testicular tumour cells would similarly express the various alternatively spliced transcripts of Ang-1. After normalization of cDNA samples

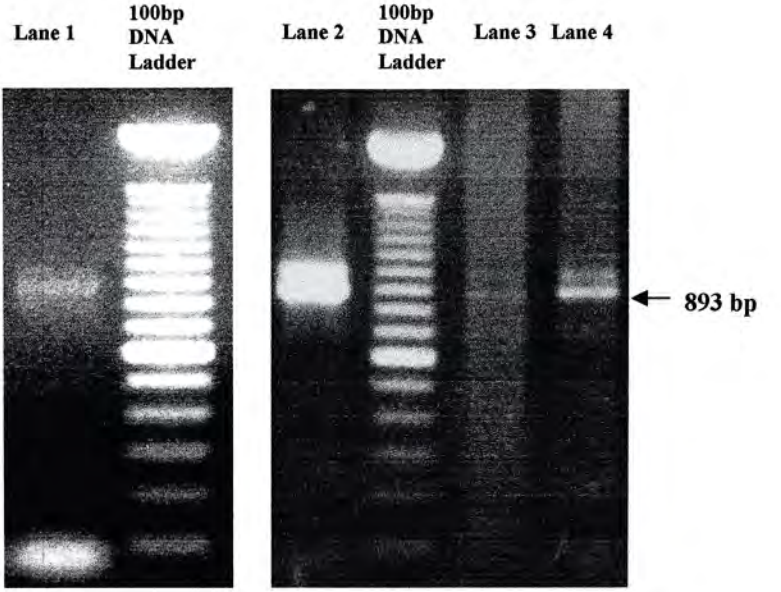
using β-actin primers, it could be noted that PCR product for β-actin was visible in all lanes and the product bands were of comparable intensities in all cDNA samples. In Figure 3.4, the results seen by RT-PCR analysis revealed the correctly sized PCR product only for the full length Ang-1.5 kb (1013 bp) in adult rat testis, C6 rat glioma cells, rat testicular blood vessel, primary germ cells, primary Sertoli cells, mouse testis as well as LC540, MLTC-1 and R2C testicular cell lines but not in TM3 and TM4 testicular cell lines. These seem to be contradictory to the detection of Ang-1 expression in TM3 and TM4 using nested PCR approach as mentioned in Section 3.1.2. A possible explanation of these observations was the relatively lower sensitivity of RT-PCR when compared with nested PCR approach.

Although no Ang-1 isoforms could be detected in primary testicular cells and other testicular cell lines using Ang-1 (S1+AS3) primer pair, it was possible that other Ang-1 primer pairs were able to detect the presence of these isoforms in testis. Therefore, RT-PCR coupled with another primer pair was employed. Ang-1 (S1+AS2) primer pair was used to detect the 0.9-kb and 1.5-kb transcripts but no 254-bp product (corresponding to the 0.9-kb isoform) was detected, regardless of the magnesium chloride concentration and primer annealing temperature being tested in the PCR reaction (as shown in Figure 3.5). Only an 893-bp product band corresponding to the presence of Ang-1.5kb transcript was amplified. Again, in all



Concentration of magnesium chloride: 1.5 mM
PCR condition: 94 °C for 1 minute; 60 °C for 45 seconds; 72 °C for 1 minute 30 seconds for 35 cycles.
Primers: sense (S1): 5'-CCGGATTTCTCTTCCCAGAAAC-3' (nt. 227-248)
antisense (AS3): 5'-CCTGCTGTCCCTGTGTGACCTTTT-3' (nt. 1216-1239)
(GenBank Accession No.: U83509)

Figure 3.4 Detection of the four alternatively spliced species of Ang-1 mRNA in the adult rat testis and testicular cell types using RT-PCR. PCR products were separated in a 1.2% ethidium bromide-stained DNA agarose gel. The positive bands occur at the expected size of 1013 bp which corresponds to the presence of transcripts for the full-length form of Ang-1 (i.e. Ang-1.5 kb). PCR products that correspond to alternatively spliced transcripts for the shorter forms of Ang-1 (i.e. Ang-1.3 kb, Ang-0.9 kb and Ang-0.7 kb) are absent. The lower panel shows the corresponding signal intensity for β-actin using the same amount of template.



Lane 1: 1.5 mM magnesium chloride; primer annealing temperature of 58 °C.
Lane 2: 1.5 mM magnesium chloride; primer annealing temperature of 55 °C.
Lane 3: 2.0 mM magnesium chloride; primer annealing temperature of 55 °C.
Lane 4: 2.5 mM magnesium chloride; primer annealing temperature of 55 °C.
PCR condition: 94 °C for 1 minute; 55 °C or 58 °C for 45 seconds; 72 °C for 1 minute 30 seconds for 35 cycles.
Primers: sense (S1): 5'-CCGGATTTCTCTTCCCAGAAAC-3' (nt. 227-248)
antisense (AS2): 5'-AGCATGTACTGCCTCTGACTGGT-3' (nt. 1097-1119)
(GenBank Accession No.: U83509)

Figure 3.5 Detection of alternatively spliced species of Ang-1 mRNA (Ang-1.5kb and Ang-0.9kb) in the adult rat testis using RT-PCR. PCR products were separated in a 1.2% ethidium bromide-stained DNA agarose gel. The positive bands (indicated by the arrow) occur at the expected size of 893 bp, which correspond to the presence of transcripts only for full-length form of Ang-1 (i.e. Ang-1.5 kb). The PCR product that corresponds to the Ang-0.9 kb isoform having the expected size of 254 bp is absent.

testicular cell types or cell lines analyzed (as shown in Figure 3.4), no expression of other alternatively spliced Ang-1 transcripts was found with Ang-1 (S1+AS2) primer pair (Figure 3.5), thus strongly suggesting that the full-length Ang-1.5 kb is the only or the most abundant form of Ang-1 in testis. Taken together, only Ang-1.5 kb (full-length) was detected in adult rat testis.

3.2 Expression of Ang-2 and Ang-2 isoforms in the testis and various testicular cell types

3.2.1 Detection of Ang-2 expression in the testis and testicular cell types by nested PCR

Nested PCR analysis was used to demonstrate the expression of Ang-2 in the testis and several testicular cell types. Primers with 1-2 degenerated nucleotide bases were designed based on a highly conserved region of the mouse and human Ang-2 mRNA. The product from the first stage of the PCR reaction was diluted to an overall ratio of 1/500 for the subsequent second stage of the PCR reaction. C6 rat glioma cells were included in this experiment as a positive control. After nested PCR amplification, a product corresponding to the expected size of 443 bp was amplified from adult rat testis, primary Leydig cells, primary Sertoli cells, primary germ cells, rat testicular blood vessel, mouse testis and C6 rat glioma cells. Representative results of the nested PCR are shown in Figure 3.6.

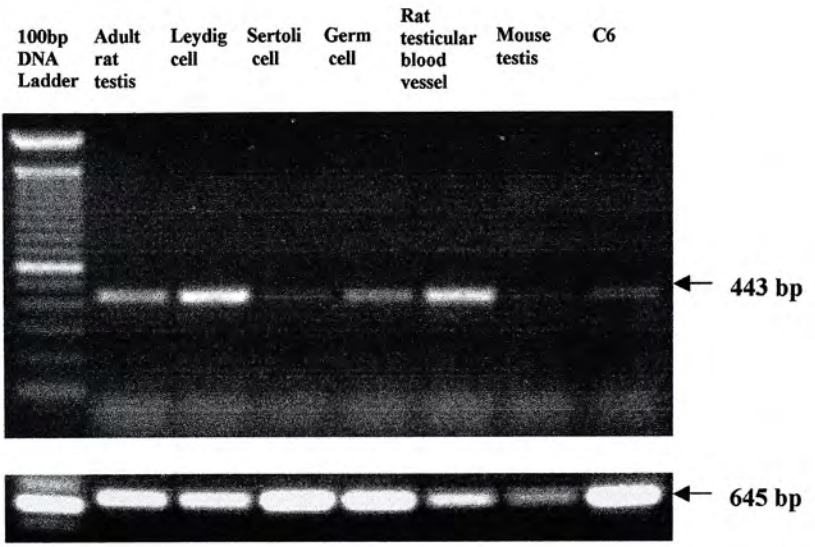


Figure 3.6 Analysis of Ang-2 expression in the adult rat testis and primary rat testicular cells using nested PCR. Products from the second stage of the nested PCR were separated in a 1.2% ethidium bromide-stained DNA agarose gel. The positive bands (indicated by the arrow) correspond to the product of interest having the expected size of 443 bp for Ang-2. The lower panel shows the corresponding signal intensity for β-actin using the same amount of template.

3.2.2 Detection of Ang-2 expression in testicular cell lines by nested PCR

As a next step, Ang-2 expression was investigated in testicular cell lines using nested PCR approach with the same primer pair as the one described in Section 3.2.1. The product from the first stage of the PCR reaction was diluted to a ratio of 1/500 for the subsequent second stage of the PCR reaction. The expression pattern of Ang-2 in testicular cell lines was found to be different from that of Ang-1. As shown in Figure 3.7, faint bands were observed in TM3 and TM4 cells but not other testicular cell lines. They corresponded to the expected size of PCR product (i.e. 443 bp) from the amplification of Ang-2 mRNAs. The amplification of β-actin was used as the control for comparable amount and quality of mRNA among samples. After the first stage of the PCR reaction, the product bands for β-actin were of comparable intensities in all samples. Before nested PCR was carried out, a single PCR reaction with the external primers was performed several times on testicular cell lines using cDNA from the same preparation of total RNA used for subsequent experiments. But Ang-2 mRNA expression was not detectable in TM3 cells even after 40 cycles of PCR amplification (data not shown). Therefore, Ang-2 mRNA could be demonstrated in TM3 mouse Leydig cells only by the nested PCR approach. With the primer pair used in this part of the study, product bands corresponding to the expected size of 443 bp were readily detected in adult rat testis

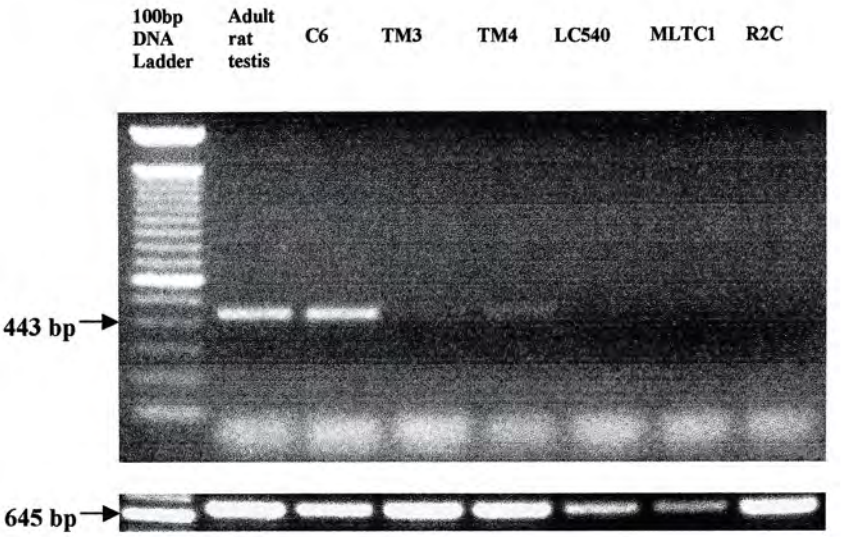


Figure 3.7 Analysis of Ang-2 expression in testicular cell lines using nested PCR. Products from the second stage of the PCR reaction were separated in a 1.2% ethidium bromide-stained DNA agarose gel. The positive bands (indicated by the arrow) correspond to the product of interest having the expected size of 443 bp for Ang-2. The lower panel shows the corresponding signal intensity for β-actin using the same amount of template.

and C6 rat glioma cells (used as a positive control). Ang-2 transcripts could not be detected in other testicular cell lines such as LC540, MLTC-1 and R2C testicular cells. In general, the results obtained in Sections 3.2.1 and 3.2.2 suggested that the Ang-2 mRNA expression was detected in Sertoli cells, Leydig cells and possibly germ cells of the testis.

3.2.3 Sequence analysis of Ang-2 transcript amplified from adult rat testis

At the start of the study, the presence of Ang-2 transcripts in the rat testis was confirmed by sequencing the product from PCR reaction using a pair of primers which gave reasonably strong signal for the rat testis. However, this primer pair was different from the ones used in subsequent parts of the study. So strictly speaking, the sequencing result from the testis could not be applied to confirm the authenticity of the nested PCR products shown in Figure 3.6 and Figure 3.7 (Sections 3.2.1 and 3.2.2). For the generation of PCR product used in the sequence analysis of Ang-2 cDNA fragment from adult rat testis, the upstream primer sequence was 5'-CTGATGTGGAAGCCCAAGTACTAA-3' (S1), corresponding to 638-661 bp, whereas the downstream primer sequence was 5'-GTCACAGTAGGCCTTGATCTC-3' (AS1), corresponding to 1132-1152. The primer sequences were numbered according to the mouse Ang-2 cDNA sequence

submitted to GenBank with the following accession number of #AF004326. The concentration of magnesium chloride used in the PCR reactions was 1.2 mM. The PCR parameters were denaturation at 94 °C for 1 minute, primer annealing at 55 °C for 45 seconds and extension at 72 °C for 1 minute 30 seconds for 35 cycles. Using this set of primers, the PCR product for Ang-2 was located at 515 bp.

Using the BLAST search, it can be seen that there was a considerable degree of similarities in the region of the Ang-2 transcript that was amplified from the rat (and in this particular case, from the rat testis) when compared that reported in the mouse (92.7%) and human (82.7%) (as shown in Figure 3.8). Alignment of the known nucleotide sequence of mouse Ang-2 with the nucleotide sequence of PCR product amplified from adult rat testis showed that the mouse Ang-2 and rat Ang-2 gene sequence are highly conserved.

3.2.4 Detection of the expression of Ang-2 isoforms in adult rat testis

Three isoforms of Ang-2 (Ang-2A, Ang-2B and Ang-2C) was reported in immature chicken testis, adult quiescent chicken testis and regressed adult chicken testis [Mezquita *et al.*, 1999; Mezquita *et al.*, 2000]. Also, a cDNA encoding a novel shorter form of Ang-2 (Ang-2_{443}) was isolated from human umbilical vein endothelial cell cDNA [Kim *et al.*, 2000a]. In the present study, a specific set of

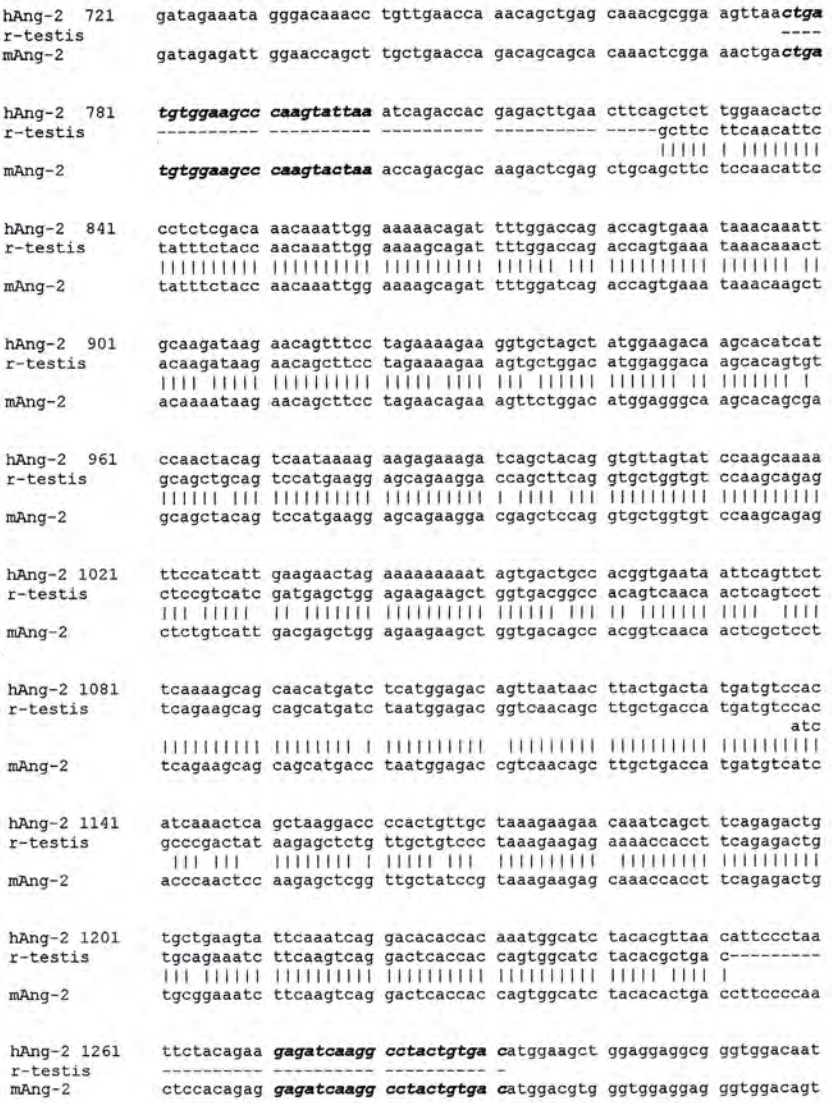
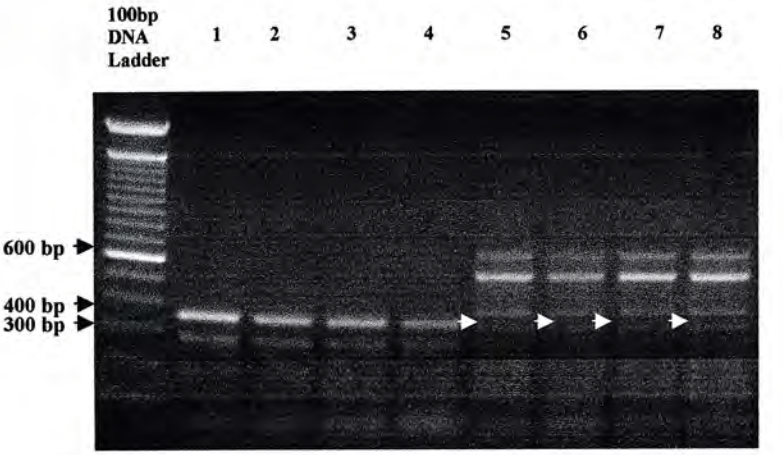


Figure 3.8 Comparing the nucleotide sequence of the PCR product obtained using specific Ang-2 primers and cDNA from adult rat testis as template, against the corresponding regions of the published mouse and human Ang-2 cDNA sequence. mAng-2, and hAng-2 represent the published sequence of mouse and human Ang-2, respectively (GenBank Accession Numbers - mAng-2: AF004326 and hAng-2: AF004327). r-testis represents the sequencing result of the PCR product amplified from adult rat testis using Ang-2 (S1+AS1) primers. The primer sequences are shown in italics and boldface. The numbering of nucleotide bases for hAng-2 is shown to the left of the sequence.

Ang-2 (S3+AS4) primers with 1-2 degenerated nucleotide bases was designed based on highly conserved regions of the mouse and human Ang-2 cDNA sequences to investigate whether the different Ang-2 mRNA isoforms could be expressed in adult rat testis (Figure 2.4). The primer sequences were numbered according to the mouse Ang-2 cDNA sequence submitted to GenBank with the following accession number of #AF004326.

First of all, a much-relaxed PCR condition (2.5 mM magnesium chloride and primer annealing temperature of 55 °C) was used for amplification of template from adult rat testis. The band of interest (indicating the possible presence of Ang-2_{443} as found in human) with a size close to 409 bp was excised from the gel for purification (Figure 3.9) and subjected to a second PCR amplification using the same PCR condition and the same set of primers (S3+AS4). The purified product from the first stage of the PCR reaction was diluted to an overall ratio of 1/100 (Lane 1) and 1/1000 (Lane 2) for the second PCR amplification. After the second PCR amplification, a major band slightly smaller than the expected size (409 bp) and an additional smaller band were found (data not shown). Although the main product band was found not to match the expected size of Ang-2_{443} in human, it was possible that other splice variants of Ang-2 in the rat could produce a RT-PCR product of a different size. Automated DNA sequencing analysis was therefore



Lane 1 to 4: Ang-2 (S3+AS3) primer pair
Lane 5 to 8: Ang-2 (S3+AS4) primer pair
Concentration of magnesium chloride: Ang-2 (S3+AS3) primer pair: 2.5 mM
Ang-2 (S3+AS4) primer pair: 2.5 mM
PCR condition: Ang-2 (S3+AS3) primer pair: 94 °C for 1 minute; 58 °C for 45 seconds; 72 °C for 1 minute 30 seconds for 25 cycles.
Ang-2 (S3+AS4) primer pair: 94 °C for 1 minute; 55 °C for 45 seconds; 72 °C for 1 minute 30 seconds for 25 cycles.

Ang-2 (S3+AS3) primer pair:
sense (S3): 5'-TGTCCAATGC(C/T)GTGCAGAGGGA-3' (nt. 392-413)
antisense (AS3): 5'-CACTGGTCTG(A/G)TCCAAAATCTG-3' (nt. 727-748)
Ang-2 (S3+AS4) primer pair:
sense (S3): 5'-TGTCCAATGC(C/T)GTGCAGAGGGA- 3' (nt. 392-413)
antisense (AS4): 5'-TCATG(C/T)TGCTGCTTCTGAAG-3' (nt. 940-959)
(GenBank Accession No.: AF004326)

Figure 3.9 Detection of alternatively spliced species of Ang-2 mRNA in the adult rat testis using RT-PCR. PCR products were separated in a 1.2% ethidium bromide-stained DNA agarose gel. Lanes 1-4 show the use of cDNA from the adult rat testis as template and Ang2 (S3+AS3) as primers. Lanes 5-8 show the use of the same template but Ang2 (S3+AS4) as primers. In both cases, only the products that correspond to the expected sizes for the full-length Ang-2 transcript are present - 357 bp when using Ang2 (S3+AS3) primers and 568 bp when using Ang2 (S3+AS4) primers. PCR products that correspond in size to the truncated form of Ang-2 are absent – 198 bp for Ang2 (S3+AS3) primers and 409 bp for Ang2 (S3+AS4) primers. Also the absence of the truncated form of Ang-2 was confirmed by sequencing the PCR products that appeared close enough in the expected sizes, as indicated by white arrowheads.

performed to study the sequence of the major band but not the weaker and smaller band. Sequence analyses indicated that the main product was not Ang-2 or other Ang-2 isoforms (data not shown).

3.3 Expression of Ang-3 in the testis and testicular cell types

3.3.1 Detection of Ang-3 expression in the testis and primary testicular cells by RT-PCR

This part of the study deals with the expression of Ang-3 in the testis, since Ang-4 is expressed in human while its counterpart in mice (and possibly also in other rodents such as the rat) is Ang-3 [Valenzuela *et al.*, 1999]. It is important to note that so far there has been no report on the expression of Ang-3 in other species besides the mouse.

Since in the mouse, Northern blot analysis showed high level of Ang-3 expression in the lung, the same organ from adult rats was chosen as a positive control for RT-PCR analysis of Ang-3 expression in the testis and other testicular cell types [Valenzuela *et al.*, 1999; Brown *et al.*, 2000].

The primer pair was designed based on the published mouse Ang-3 cDNA sequences. Positive Ang-3 expression was found in all cDNA preparations being investigated. As shown in Figure 3.10, Ang-3 was expressed though poorly in adult rat testis, primary Leydig cells, primary Sertoli cells, primary germ cells

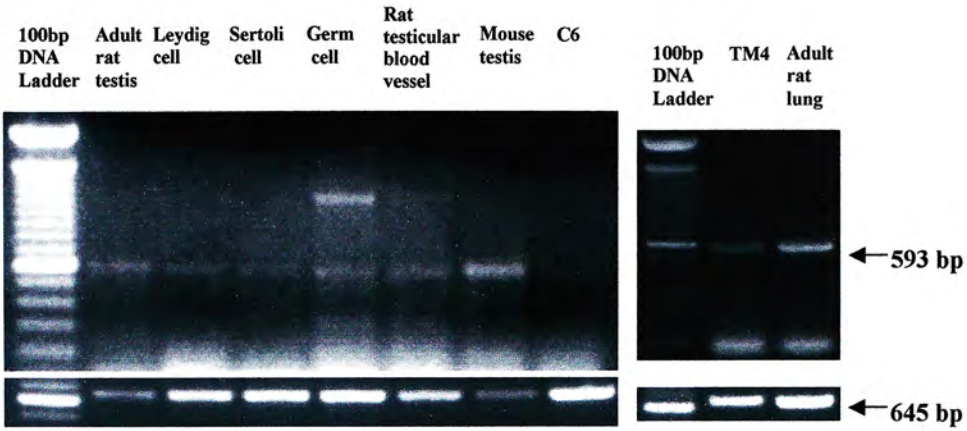


Figure 3.10 Analysis of Ang-3 expression in the adult rat testis and primary rat testicular cells using RT-PCR. PCR products were separated in a 1.2% ethidium bromide-stained DNA agarose gel. The positive bands (indicated by the arrow) correspond to the product of interest having the expected size of 593 bp for Ang-3. The lower panel shows the corresponding signal intensity for β-actin using the same amount of template.

and rat testicular blood vessel. Of interest, a band of 593 bp, thus corresponding in size to the RT-PCR product expected from mouse Ang-3 mRNA, was more readily detectable in mouse testis. In contrast, the expression of Ang-3 was undetectable in C6 rat glioma cells. All of these cDNA preparations also an expected PCR product for β-actin with comparable band intensity, indicating successful RNA extraction, reverse transcription and comparable amounts of the templates being used.

3.3.2 Detection of Ang-3 expression in testicular cell lines by RT-PCR

RT-PCR analysis was performed on five testicular cell lines to determine which of the various testicular cell lines expressed Ang-3. The same set of primers mentioned in Section 3.3.1 was also applied in this part of the study. Similar to the results obtained from the study of primary Leydig cells, the levels of Ang-3 expression were also low in Leydig cell lines, including TM3, LC540, MLTC-1 and R2C testicular cells. The results were shown in Figure 3.11. Among all testicular cell lines being investigated, Ang-3 mRNA expression was only easily detectable in TM4 mouse Sertoli cells at relatively higher levels.

3.3.3 Sequence analysis of Ang-3 transcripts amplified from TM4 mouse Sertoli cells and adult rat testis

To demonstrate whether Ang-3 transcripts amplified from TM4 mouse

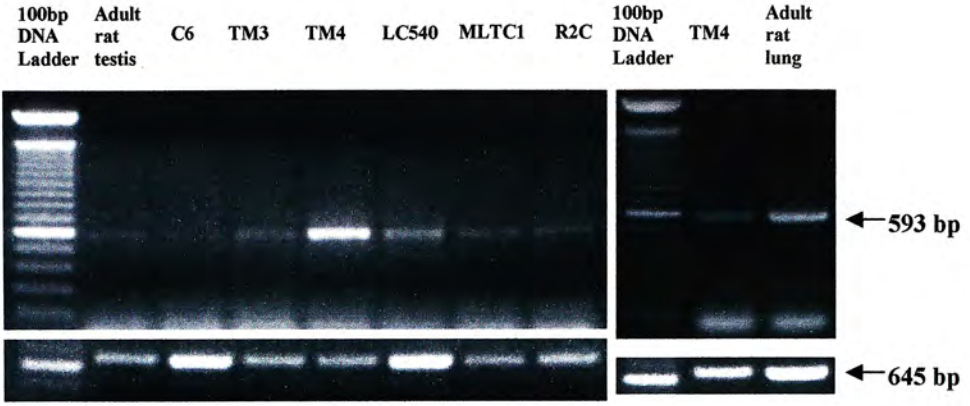
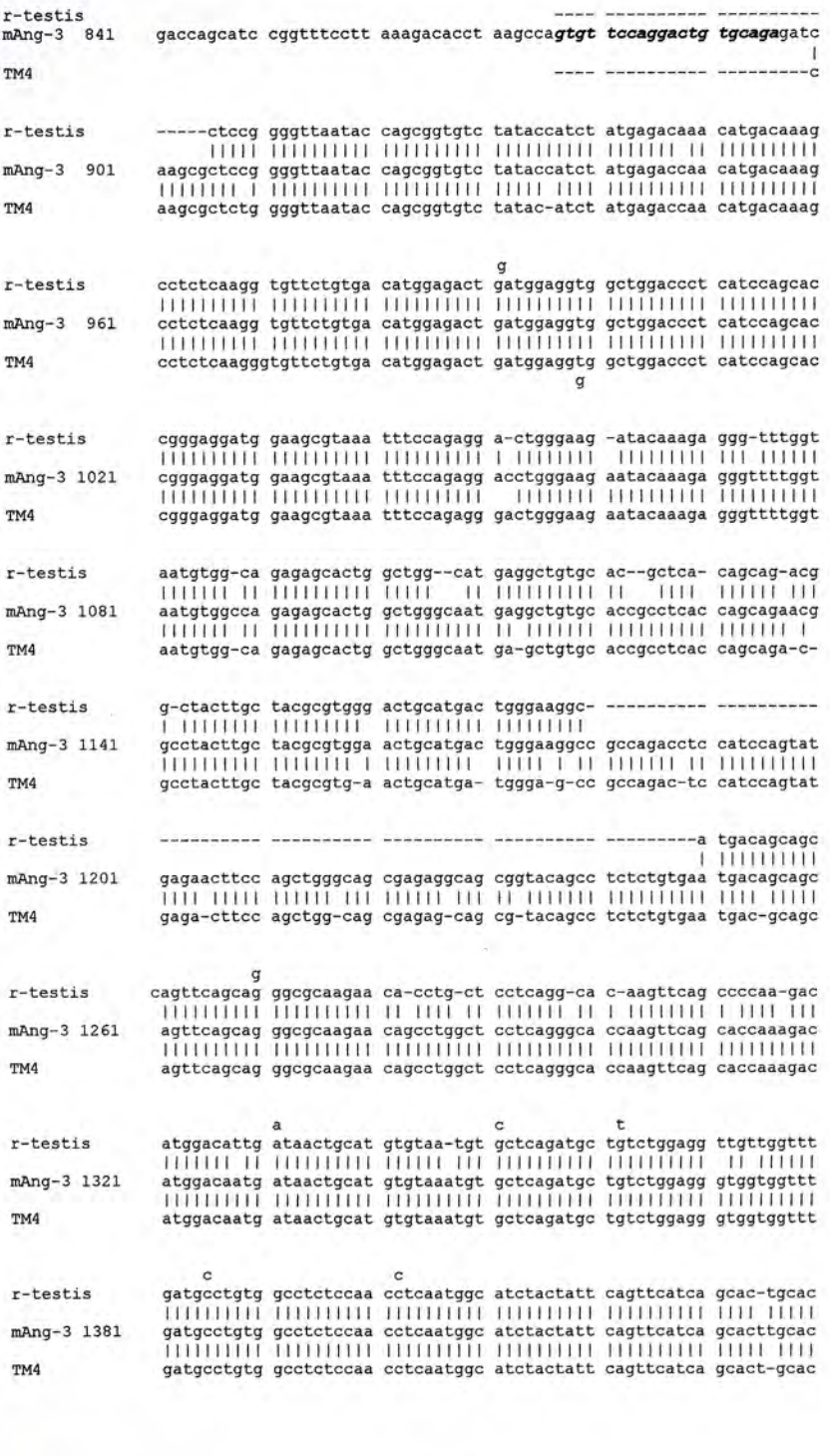


Figure 3.11 Analysis of Ang-3 expression in testicular cell lines using RT-PCR. PCR products were separated in a 1.2% ethidium bromide-stained DNA agarose gel. The positive bands (indicated by the arrow) correspond to the product of interest having the expected size of 593 bps for Ang-3. The lower panel shows the corresponding signal intensity for β-actin using the same amount of template.

Sertoli cells actually correspond to mouse counterpart of Ang-3 cDNA, automatic DNA sequencing analysis was performed using the same set of primer for generating the PCR product (as mentioned in Sections 3.3.1 and 3.3.2). Similarly, RT-PCR revealed the correct size PCR product for Ang-3 (593 bp) in adult rat testis. To confirm the identity of this PCR product from adult rat testis, automatic DNA sequencing analysis was performed using the same set of primer for generating the PCR product.

By searching Standard Nucleotide-nucleotide BLAST Database as described under Materials and Methods (Section 2.3.7), a high degree of identity was found with cDNA generated from TM4 mouse Sertoli cells and the known partial nucleotide sequence of mouse Ang-3. The nucleotide sequence of Ang-3 from TM4 mouse Sertoli cells was found to share 96.8% identity with the corresponding region of the mouse Ang-3 DNA sequence when the extra nucleotide residues present in the sequence of TM4 mouse Sertoli cells were ignored in the calculations (as shown in Figure 3.12). Therefore, the PCR products obtained in Sections 3.3.1 and 3.3.2 could be proved to be the authentic fragments of Ang-3.

Using the BLAST search, it can be seen that a relatively high degree of identity was found with template amplified from adult rat testis and known partial


r-testis
mAng-3 841
TM4
r-testis
mAng-3 901
TM4
r-testis
mAng-3 961
TM4
r-testis
mAng-3 1021
TM4
r-testis
mAng-3 1081
TM4
r-testis
mAng-3 1141
TM4
r-testis
mAng-3 1201
TM4
r-testis
mAng-3 1261
TM4
r-testis
mAng-3 1321
TM4
r-testis
mAng-3 1381
TM4

```
r-testis        aagatcaa-- ---------- ---------
                ||||||||
mAng-3 1441     aagatcaatg gcatccgctg gcactacttc cgaggcccca gctactcact gcacggcaca
                ||||||||
TM4             aagatcaa-- ---------- ---------


r-testis
mAng-3 1501     cgcatgatgc tgaggccaat gggtgcctga
TM4
```

Figure 3.12 Comparing the nucleotide sequence of PCR products obtained using specific Ang-3 primers and cDNAs from TM4 mouse Sertoli cells and adult rat testis as templates, against known partial sequence of mouse Ang-3. mAng-3 represents the published sequence of mouse Ang-3 (GenBank Accession Number - mAng-3: AF113707). TM4 and r-testis represent the nucleotide sequence of PCR products from TM4 mouse Sertoli cells and adult rat testis, respectively. The present data show the first partial nucleotide sequence of rat Ang-3. Primer sequences are shown in italics and boldface. Gaps between nucleotide bases are indicated by dashes. Extra nucleotide bases were shown above and below the sequence for adult rat testis and TM4 mouse Sertoli cells, respectively. The numbering of nucleotide bases for mAng3 is shown to the left of each sequence.

nucleotide sequence of mouse Ang-3. The nucleotide sequence of Ang-3 from adult rat testis was found to be 94.3% identical to the corresponding region of the mouse Ang-3 DNA sequence when the extra nucleotide residues present in the sequence of template from adult rat testis were ignored in the calculations (as shown in Figure 3.12). Alignment of the known nucleotide sequence of mouse Ang-3 with the nucleotide sequence of PCR product amplified from adult rat testis indicated that the nucleotide sequences of mouse Ang-3 and rat Ang-3 are highly conserved. This study represented the first to report on the presence of the nucleotide sequence of Ang-3 in the rat species.

3.4 Expression of Tie 1 and Tie 2 in the testis and testicular blood vessel

3.4.1 Detection of Tie 1 and Tie 2 expression in the testis and rat testicular blood vessel by RT-PCR

The expression of Tie 1 and Tie 2 was investigated in adult rat testis and rat testicular blood vessel. RT-PCR was performed using Tie 1 and Tie 2 specific primers to cDNA prepared from adult rat testis and isolated testicular subcapsular artery. Representative experiments were shown in Figures 3.13 and 3.14. Specific PCR products of Tie 1 and Tie 2 were noted in adult rat testis and rat testicular blood vessel. The PCR bands were located at 425 bp for Tie 1 and 457 bp for Tie 2. The expression pattern of Tie 1 was the same as that of Tie 2. The identities of Tie

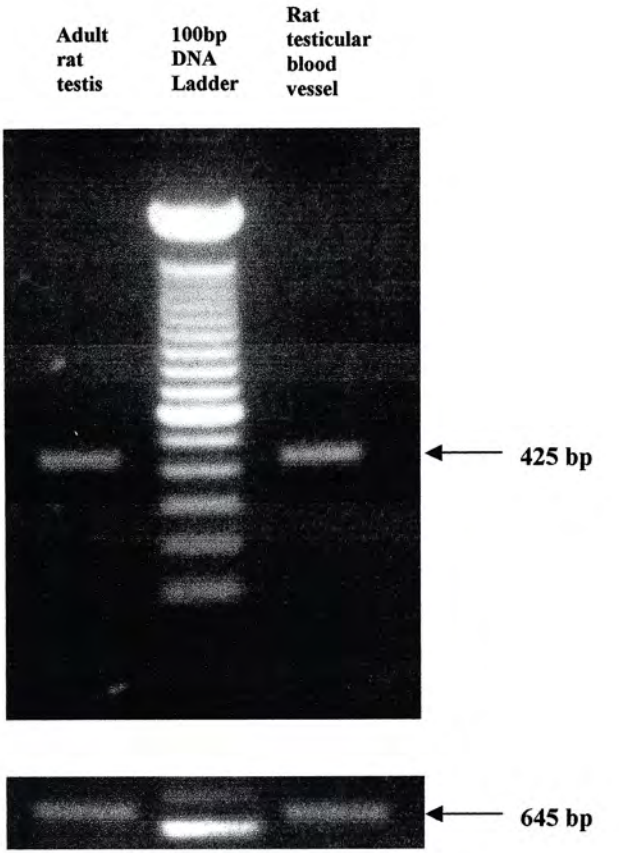


Figure 3.13 Analysis of Tie 1 expression in the adult rat testis and testicular blood vessel using RT-PCR. PCR products were separated in a 1.2% ethidium bromide-stained DNA agarose gel. The positive bands correspond to the product of interest having the expected size of 425 bps for Tie 1. The lower panel shows the corresponding signal intensity for β-actin using the same amount of template.

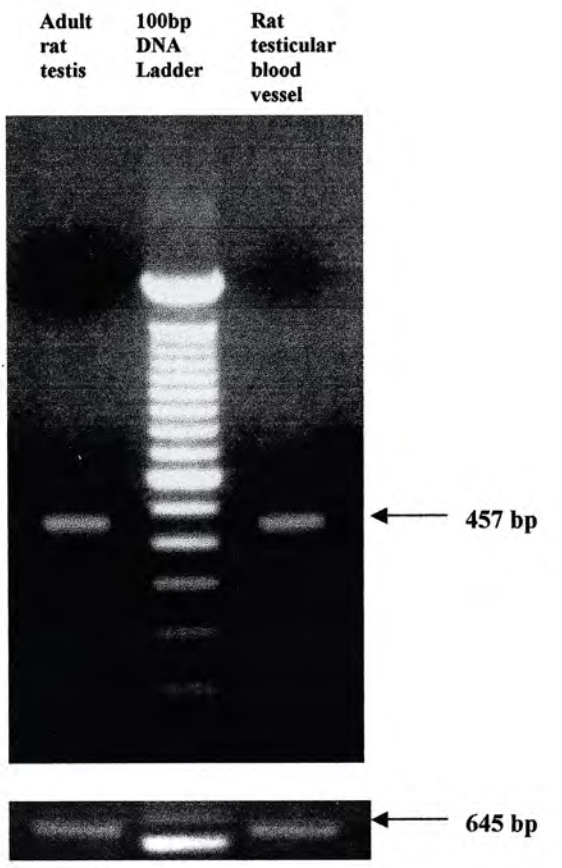


Figure 3.14 Analysis of Tie 2 expression in the adult rat testis and testicular blood vessel using RT-PCR. PCR products were separated in a 1.2% ethidium bromide-stained DNA agarose gel. The positive bands correspond to the product of interest having the expected size of 457 bps for Tie 2. The lower panel shows the corresponding signal intensity for β-actin using the same amount of template.

1 and Tie 2 transcripts amplified from adult rat testis and rat testicular blood vessel were confirmed by direct sequencing of PCR products.

3.4.2 Sequence analysis of Tie 1 transcripts amplified from adult rat testis and rat testicular blood vessel

To confirm the authenticity of the PCR products obtained from RT-PCR analysis coupled with Tie 1 primers in Figure 3.13, direct sequencing of the PCR-amplified products from adult rat testis and rat testicular blood vessel was performed.

By searching Standard Nucleotide-nucleotide BLAST Database as described under Materials and Methods (Section 2.3.7), there was a considerable degree of identity between Tie 1 transcripts amplified from adult rat testis or rat testicular blood vessel and the corresponding region of the mouse Tie 1 DNA sequence (as shown in Figure 3.15). Also, Tie 1 transcripts amplified from adult rat testis and rat testicular blood vessel had retained similarity to both mouse Tie 1 and human Tie 1, suggesting a conserved functional role of Tie 1 in these species.

3.4.3 Sequence analysis of Tie 2 transcripts amplified from rat testicular blood vessel

In order to determine whether the RT-PCR products shown in Figure 3.14 actually corresponds to rat Tie 2 cDNA, DNA sequencing analysis was performed

Figure 3.15 Comparing the nucleotide sequence of PCR products obtained using specific Tie 1 primers and cDNA from adult rat testis and rat testicular blood vessel as templates, against known sequences of mouse and human Tie 1. mTie 1 and hTie 1 represent the published sequences of mouse and human Tie 1, respectively (GenBank Accession Numbers - mTie 1: X80764 and hTie 1: X60957 S89716). r-testis and r-TBV represent the nucleotide sequence of PCR products from adult rat testis and rat testicular blood vessel, respectively. Primer sequences are shown in italics and boldface. 'n' represents the unknown nucleotide base in the sequencing results. Gaps between nucleotide bases are indicated by dashes. Extra nucleotide bases are shown above and below the sequence for adult rat testis and rat testicular blood vessel, respectively. The numbering of nucleotide bases for mTie 1 is shown to the left of the sequence.

```
r-testis                - ---------- ---------- ---------- gccacagagc tggagttcaa
                                                           |||||||||  ||||||||||
mTie 1 1381     ctgtgaaaag tcagaccgga tcccccagat cctcagtatg gccacagagg tggagttcaa
                                                                     |||||||
r-TBV                   - ---------- ---------- ---------- ---------- ---agttcaa
hTie 1          ctgtgagaag tcagaccgga tcccccagat cctcaacatg gcctcagaac tggagttcaa


r-testis        cttagggaca atgccccgga tcaactgtgc agctgccggg aatcccttcc cagtacgggg
                | |||||||  |||||||||| |||||||||| |||||||||| |||||||||| |||| |||||
mTie 1 1441     catagggacg atgccccgga tcaactgtgc agctgccggg aatcccttcc cagtgcgggg
                |||||||||  |||||||||| |||||||||| |||||||||| ||||| |||| |||| |||||
r-TBV           catagggaca atgccccgga tcaactgtgc agctgccggg aatcc-ttcc cagtacgggg
hTie 1          cttagagacg atgccccgga tcaactgtgc agctgcaggg aaccccttcc ccgtgcgggg


r-testis        cagcatggaa ctccgcaagc cagacggcac catgcttctg tctaccaaag ccattgtgga
                ||||||| || || ||||||| |||| ||||| ||||||| || |||||||||| |||||||||
mTie 1 1501     cagcatgaaa cttcgcaagc cagatggcac catgcttttg tctaccaaag tcattgtgga
                ||||||| || || ||||||| |||| ||||| ||||||| || |||| ||||| |||||||||
r-TBV           cagcatggaa ctccgcaagc cagacggcac catgcttctg tcta-caaag ccattgtgga
hTie 1          cagcatagag ctacgcaagc cagacggcac tgtgctcctg tccaccaagg ccattgtgga


                                t      c                       t
r-testis        gccagatagg accacagctg agtttggagt gccccgtttg actcttgggg acagcgggtt
                ||| || ||| |||||||| | |||||||||| |||| ||||| |||||||||| |||| |||||
mTie 1 1561     gccagacagg accacagcag agtttgaagt gcccagtttg actcttgggg acagtgggtt
                |||||| ||| |||||||| | |||| || || |||| ||||| |||||||||| |||| |||||
r-TBV           gccagatagg accacagctg agttcgaggt gccccgtttg actcttgggg acagcgggtt
hTie 1          gccagagaag accacagctg agttcgaggt gccccgcttg gttcttgcgg acagtgggtt


                tg     t                 t           t          t
r-testis        ctgggaatgc cgtgtatcga cgtctggtgg ccaagaaagc cggcgcttca aggtcaatgt
                |||||||||| || ||||||| | |||||||| |||||| ||| |||||||||| ||||||||||
mTie 1 1621     ctgggaatgc cgcgtatcga cttctggtgg ccaagacagc cggcgcttca aggtcaatgt
                |||||||||| || ||||| | | |||||||| |||||| ||| | |||||||| ||||||||||
r-TBV           ctgggaatgc cgtgtatcca cgtctggtgg ccaagatagc cngcgcttcaaaggtcaatgt
                                                            a          c
hTie 1          ctgggagtgc cgtgtgtcca catctggcgg ccaagacagc cggcgcttca aggtcaatgt


r-testis        caaagtaccc ccagtgcctc tgactgcacc tcggctcctg gccaagcaga gccgtcagct
                |||||||||| |||||||||| |||||||||| ||| |||||| |||||||||| ||||||||||
mTie 1 1681     caaagtaccc ccagtgcctt tgactgcacc tcgactcctg gccaagcaga gccgtcagct
                |||||||||| | | ||| | |||||||  || |||||| || ||||| |  ||| |||||
r-TBV           tcaaagtaccc caattgctct gaactgcact ccggctcctg gcaaagcaaaanccgccagct
                     t                                    gc
hTie 1          gaaagtgccc cccgtgcccc tggctgcacc tcggctcctg accaagcaga gccgccagct


r-testis        tgtggtctcc ccactgttct ccttcggtgg ggatggaccc ---------- ----------
                |||||||||| |||||| ||| |||| |||| ||||||||||
mTie 1 1741     tgtggtctcc ccactggtct cctttagtgg ggatggaccc atctcctctg tccgcctgca
                ||||||| || |||||||||| ||||| |||| |||||||||| |||||||||| |
r-TBV           tgtggtc-cc ccactggtct cccttcgtgg ggatggaccc atctcctctg t---------
                                              a
hTie 1          tgtggtctcc ccgctggtct cgttctctgg ggatggaccc atctccactg tccgcctgca


r-testis        ---------- ----
mTie 1 1801     ctaccggccc caggacagca cgattgcctg gtctgccatt gtggtggatc ccagtgagaa
r-TBV           ---------- ----
hTie 1          ctaccggccc caggacagta ccatggactg gtcgaccatt gtggtggacc ccagtgagaa
```

on RT-PCR product from rat testicular blood vessel using the isolation and sequencing procedure outlined under Materials and Methods (Sections 2.3.6 and 2.3.7).

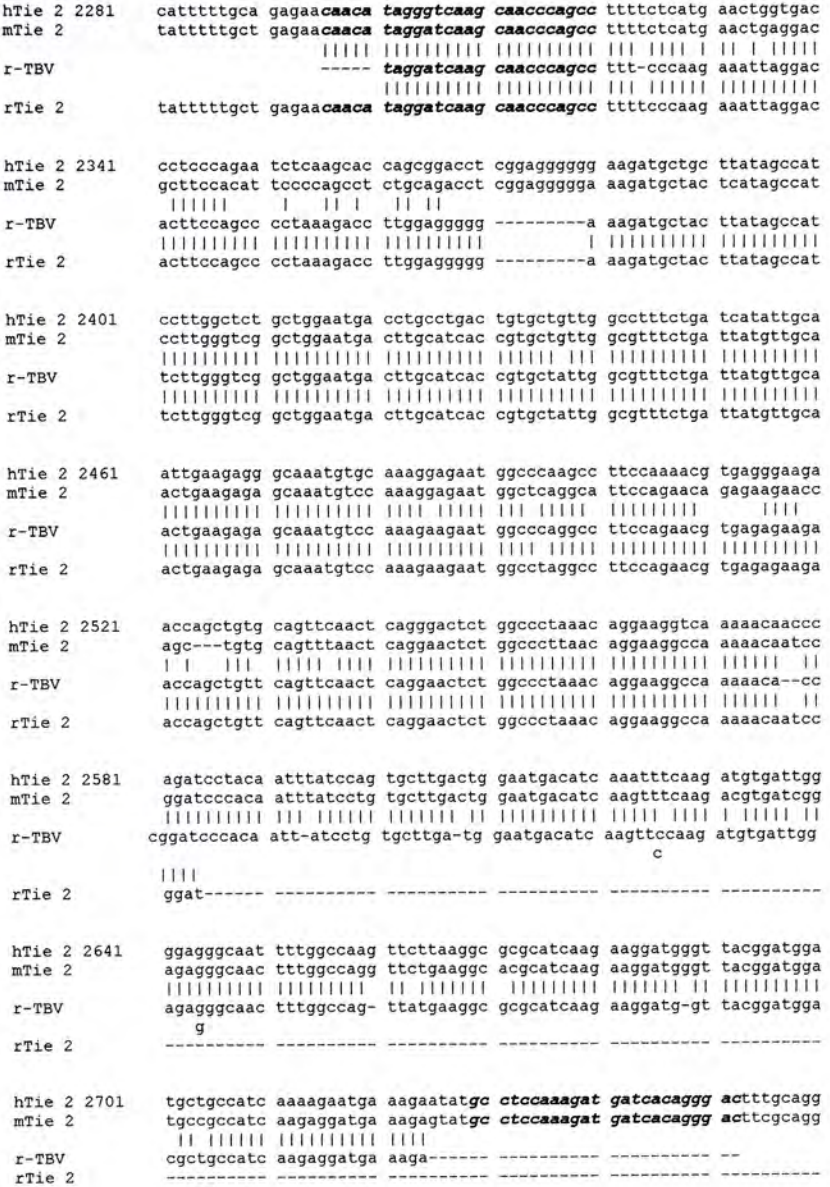
Using the BLAST search, it can be seen that there was a considerable degree of identity between the nucleotide sequence of Tie 2 transcripts amplified from rat testicular blood vessel and the corresponding region of the mouse Tie 2 DNA sequence (as shown in Figure 3.16). The nucleotide sequence of Tie 2 from rat testicular blood vessel was almost identical to the rat Tie 2 DNA sequence. Therefore, the authenticity of the RT-PCR products obtained in Section 3.41 could be confirmed and these products were rat Tie 2 DNA sequence.

Similarly, Tie 2 cDNAs amplified from rat testicular blood vessel demonstrates a high sequence identity to the nucleotide sequence of human Tie 2 within the region between the upstream primer and downstream primer. The data suggested a conserved Tie 2 nucleotide sequence across species.

3.5 Western blot analysis of Ang-1 and Ang-2 expression in testicular tissues, primary testicular cells and cell lines

In order to determine the presence of angiopoietin proteins in testis, Western blot analysis were performed (published data from C.L. Au, L. Yeung and M.C. Lui). A rabbit polyclonal antibody [Ang-1 (H-98), #sc-8357] raised against

Figure 3.16 Comparing the nucleotide sequence of PCR products obtained using specific Tie 2 primers and cDNA from rat testicular blood vessel as template against the known partial sequence of rat Tie 2 and the corresponding region of mouse and human Tie 2 cDNA sequence. hTie 2, mTie 2 and rTie 2 represent the published sequences of human, mouse and rat Tie 2, respectively (GenBank Accession Numbers - mTie-2: X71426, rTie-2: AF030423, and hTie-2: L06139). r-TBV represents the nucleotide sequence of the PCR product from rat testicular blood vessel. Primer sequences are shown in italics and boldface. Gaps between nucleotide bases are indicated by dashes. The numbering of nucleotide bases for hTie 2 is shown to the left of the sequence.


hTie 2 2281 catttttgca gagaacaaca taggtcaag caacccagcc ttttctcatg aactggtgac
mTie 2 tatttttgct gagaacaaca taggatcaag caacccagcc ttttctcatg aactgaggac
r-TBV ----- taggatcaag caacccagcc ttt-cccaag aaattaggac
rTie 2 tatttttgct gagaacaaca taggatcaag caacccagcc ttttcccaag aaattaggac
hTie 2 2341 cctcccagaa tctcaagcac cagcggacct cggaggggg aagatgctgc ttatagccat
mTie 2 gcttccacat tccccagcct ctgcagacct cggaggggga aagatgctac tcatagccat
r-TBV acttccagcc cctaaagacc ttggaggggg ---------a aagatgctac ttatagccat
rTie 2 acttccagcc cctaaagacc ttggaggggg ---------a aagatgctac ttatagccat
hTie 2 2401 ccttggctct gctggaatga cctgcctgac tgtgctgttg gcctttctga tcatattgca
mTie 2 ccttgggtcg gctggaatga cttgcatcac cgtgctgttg gcgtttctga ttatgttgca
r-TBV tcttgggtcg gctggaatga cttgcatcac cgtgctattg gcgtttctga ttatgttgca
rTie 2 tcttgggtcg gctggaatga cttgcatcac cgtgctattg gcgtttctga ttatgttgca
hTie 2 2461 attgaagagg gcaaatgtgc aaaggagaat ggcccaagcc ttccaaaacg tgagggaaga
mTie 2 actgaagaga gcaaatgtcc aaaggagaat ggctcaggca ttccagaaca gagaagaacc
r-TBV actgaagaga gcaaatgtcc aaagaagaat ggcccaggcc ttccagaacg tgagagaaga
rTie 2 actgaagaga gcaaatgtcc aaagaagaat ggcctaggcc ttccagaacg tgagagaaga
hTie 2 2521 accagctgtg cagttcaact cagggactct ggccctaaac aggaaggtca aaaacaaccc
mTie 2 agc---tgtg cagtttaact caggaactct ggcccttaac aggaaggcca aaaacaatcc
r-TBV accagctgtt cagttcaact caggaactct ggccctaaac aggaaggcca aaaaca--cc
rTie 2 accagctgtt cagttcaact caggaactct ggccctaaac aggaaggcca aaaacaatcc
hTie 2 2581 agatcctaca atttatccag tgcttgactg gaatgacatc aaatttcaag atgtgattgg
mTie 2 ggatcccaca atttatcctg tgcttgactg gaatgacatc aagtttcaag acgtgatcgg
r-TBV cggatcccaca att-atcctg tgcttga-tg gaatgacatc aagttccaag atgtgattgg
c
rTie 2 ggat------ ---------- ---------- ---------- ---------- ----------
hTie 2 2641 ggagggcaat tttggccaag ttcttaaggc gcgcatcaag aaggatgggt tacggatgga
mTie 2 agagggcaac tttggccagg ttctgaaggc acgcatcaag aaggatgggt tacggatgga
r-TBV agagggcaac tttggccag- ttatgaaggc gcgcatcaag aaggatg-gt tacggatgga
g
rTie 2 ---------- ---------- ---------- ---------- ---------- ----------
hTie 2 2701 tgctgccatc aaaagaatga aagaatatgc ctccaaagat gatcacaggg actttgcagg
mTie 2 tgccgccatc aagaggatga aagagtatgc ctccaaagat gatcacaggg acttcgcagg
r-TBV cgctgccatc aagaggatga aaga------ ---------- ---------- --
rTie 2 ---------- ---------- ---------- ---------- ---------- ----------

the amino acids 400-498 mapping at the carboxyl terminus of Ang-1 of human origin was employed. Though this antibody was raised against Ang-1, the supplier indicated that it might cross-react with Ang-2 due to close similarity of the amino acid sequence in the carboxyl termini of these two peptides. Cell lysates prepared from primary testicular cells and testicular cell lines as well as tissue lysates from adult rat testis, testicular subcapsular artery and mouse testis were used.

As shown in Figures 3.17 and 3.18, two prominent bands were consistently observed in the testis (rat and mouse), C6 rat glimoa cells (used as a positive contro), primary rat testicular cells and the various testicular cell lines, with the exception of LC540 rat Leydig tumour cells which showed only a single band. The apparent molecular weights of the two positive bands (indicated by arrowheads) were found to be 58 kDa and 72 kDa, with the former showing a stronger intensity than the latter. They correspond to the reported molecular weights for the non-glycosylated and glycosylated of Ang-1 protein. In lysates prepared from Leydig tumour cell lines (i.e. MLTC-1, LC540, R2C), C6 rat glioma cells and adult rat testis (Figure 3.17), there might be an additional positive band of 55 kDa. This would correspond to the expected molecular size for the non-glycosylated form of Ang-2 (Figure 3.19), and was detected due to the cross-reactivity of the Ang-1 (H-98) antibody to Ang-2 (as earlier pointed out). No

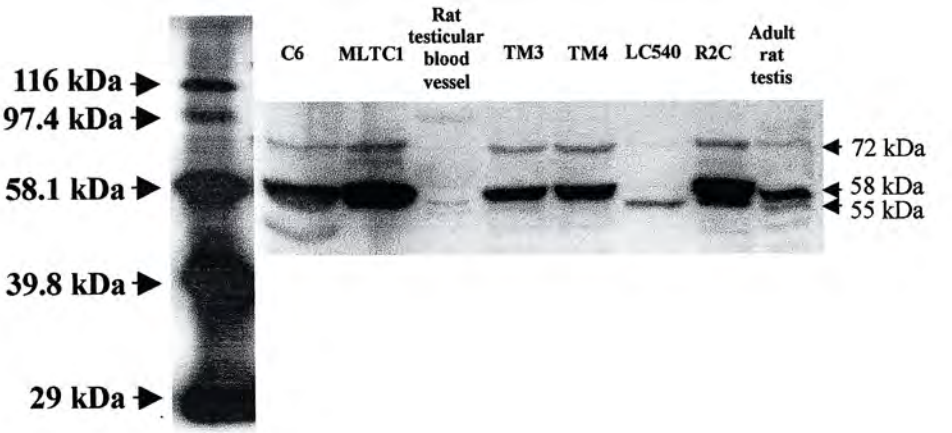


Figure 3.17 Western immunoblotting of Ang-1 (and Ang-2) in the rat testis and testicular cell lines [Lui M.C. & Au C.L.; unpublished data]. Molecular weight marker sizes shown on the left were used to estimate molecular masses. Sixty μg of proteins extracted from LC540 testicular cells and C6 rat glioma cells, 40 μg of proteins extracted from MLTC-1, R2C, TM3 and TM4 testicular cells and rat testicular blood vessel, as well as 50 μg of proteins extracted from adult rat testis were loaded onto each lane. The samples were separated by 10% SDS-PAGE and blotted. Blots were probed using a rabbit polyclonal antibody [Ang-1 (H-98), #sc-8357] (1:500) raised against Ang-1 but showed cross-reactivity to Ang-2. The protein bands that bind to the antibody was visualized using horse radish peroxidase (HRP)-conjugated protein-A secondary antibody (1:5000) and ECL. Arrowheads point to protein bands which correspond to the expected molecular sizes of Ang-1 (58 kDa, 72 kDa) and Ang-2 (55 kDa).

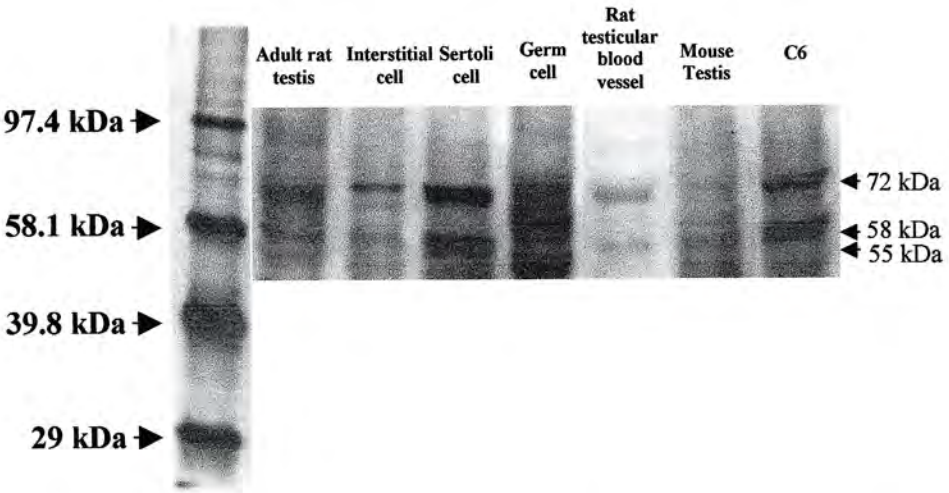


Figure 3.18 Western immunoblotting of Ang-1 (and Ang-2) in primary rat testicular cells and testicular tissues [Yeung L. & Au C.L.; unpublished data]. Molecular weight marker sizes shown on the left were used to estimate molecular masses. Sixty μg of proteins extracted from interstitial cells, germ cells, Sertoli cells, rat testicular blood vessel, adult rat testis, mouse testis and C6 rat glioma cells were loaded onto each lane. The samples were separated by 10% SDS-PAGE and blotted. Blots were probed using a rabbit polyclonal antibody [Ang-1 (H-98), #sc-8357] (1:500) raised against Ang-1 but showed cross-reactivity to Ang-2. The protein bands that bind to the antibody was visualized using horse radish peroxidase (HRP)-conjugated protein-A secondary antibody (1:5000) and ECL. Arrowheads point to protein bands which correspond to the expected molecular sizes of Ang-1 (58 kDa, 72 kDa) and Ang-2 (55 kDa, adult rat testis).

protein bands that correspond to the expected size of Ang-1 could be identified in germ cells.

Western blot analysis of Ang-2 with a specific goat polyclonal antibody [Ang-2 (C-19), #sc-7015] mapping to epitope at the carboxy terminus of human Ang-2 revealed two major protein bands of 55 kDa to 68 kDa in cell lysates prepared from C6 rat glioma cells (used as a positive control), rat and mouse testes, testicular subcapsular artery, primary rat testicular interstitial cells and Sertoli cells (Figure 3.19). These two proteins corresponded to the reported molecular weight for the non-glycosylated and glycosylated form of Ang-2. In rat testicular germ cells, no protein bands of the correct sizes could be identified. These data indicated that Ang-2 protein would occur in the testis mainly in Leydig cells, but also could be found in Sertoli cells and testicular blood vessels.

Importantly, no protein bands were seen in any of the lanes in parallel Western blots in which the blots had been incubated overnight at room temperature using PBST without the primary antibodies, thus validating the specificity of these primary antibodies.

3.6 Localization of Ang-1, Ang-2, Ang-3, Tie 1 and Tie 2 proteins in adult rat testis by immunohistochemistry

To determine if the testicular cells translate angiopoietins and Tie mRNAs,

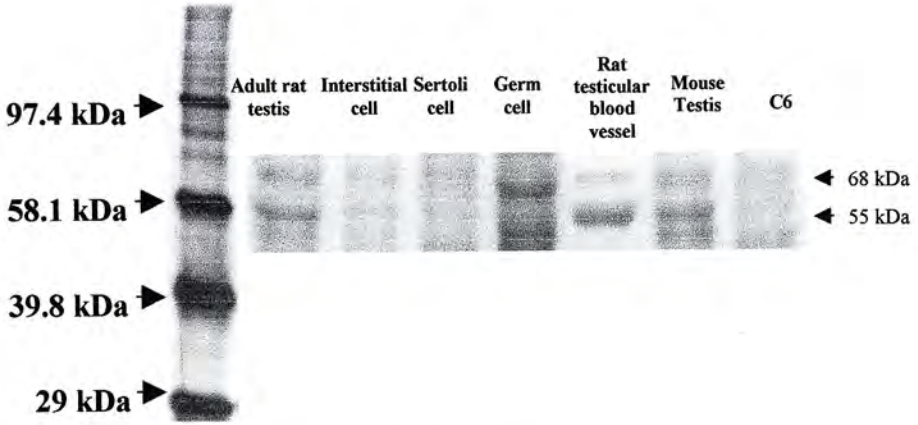


Figure 3.19 Western immunoblotting of Ang-2 in primary testicular cells and testicular tissues [Yeung L. & Au C.L.; unpublished data]. Molecular weight marker sizes shown on the left were used to estimate molecular masses. Sixty μg of proteins extracted interstitial cells, germ cells, Sertoli cells, rat testicular blood vessel, adult rat testis, mouse testis and C6 rat glioma cells were loaded onto each lane. The samples were separated by 10% SDS-PAGE and blotted. Blots were probed using a goat polyclonal antibody Ang-2 [Ang-2 (C-19), #sc-7015] (1:500) specific for Ang-2 and visualized using horse radish peroxidase (HRP)-conjugated secondary antibody (1:2500) and ECL. Arrowheads point to protein bands which correspond to the expected molecular size of Ang-2 (55 kDa, 68 kDa).

and to examine their specific sites of distribution within the testis, sections from normal adult rat testis were reacted with anti-Ang-1, anti-Ang-2, anti-Ang-1/3, anti-Tie 1 and anti-Tie 2 antibodies.

In adult rat testis, a positive immunoreactivity to Ang-1 (Figure 3.21a) and Ang-2 (Figure 3.21b) was most prominently detected in vascular smooth muscle cells and vascular endothelial cells, respectively.

Immunoreactivities for Tie 1 (Figure 3.19a) and Tie 2 (Figure 3.19b) receptors were localized mainly to vascular endothelial cells of testicular blood vessel. This was consistent with the expression of Tie mRNA in endothelial cell lines [Partanen *et al.*, 1992]. Their distributions within the testis were similarly found in the vascular endothelium.

Immunohistochemical data showed that Ang-3 was present in both the interstitial and tubular compartments of the testis as well as vascular smooth muscle cells of testicular blood vessels (as shown in Figures 3.20a & b). However in view of the fact that this particular antibody might show cross-reactivity to Ang-1, the possibility that part of the immunoreactivity might have been contributed by Ang-1 could not be entirely ruled out.

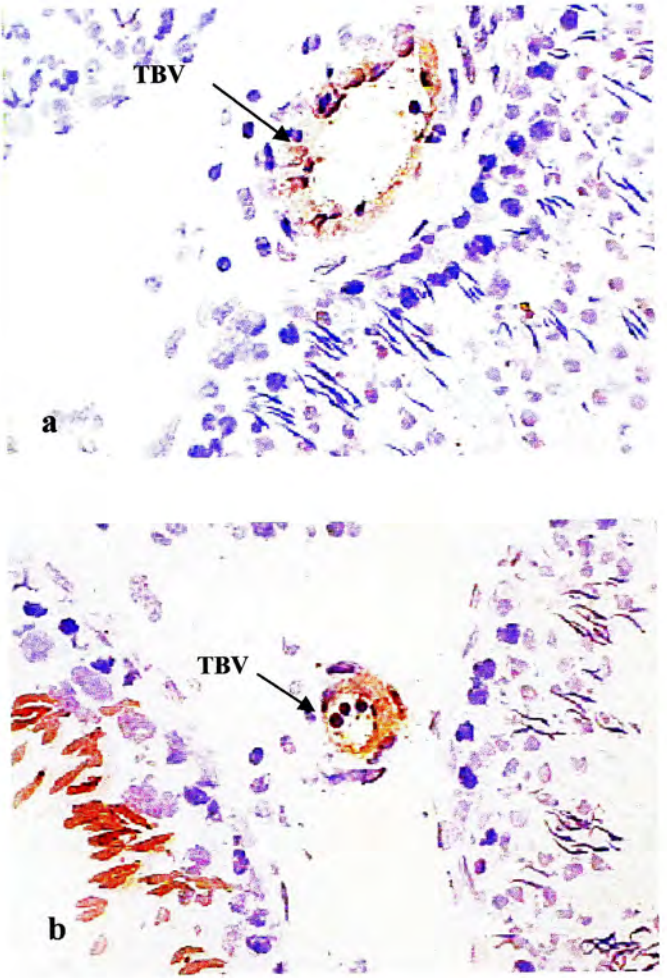


Figure 3.19 Immunohistochemical localization of Tie 1 and Tie 2 receptor tyrosine kinases on frozen sections of adult rat testes. Frozen sections were incubated with rabbit polyclonal [Tie 1 (C-18), #sc-342] and [Tie 2 (C-20), #sc-324] antibodies (1:100). Arrows indicate red immunopositive signals for (a) Tie 1 and (b) Tie 2 in rat testicular blood vessel (TBV). Magnification x400. Sections were counterstained with haematoxylin.

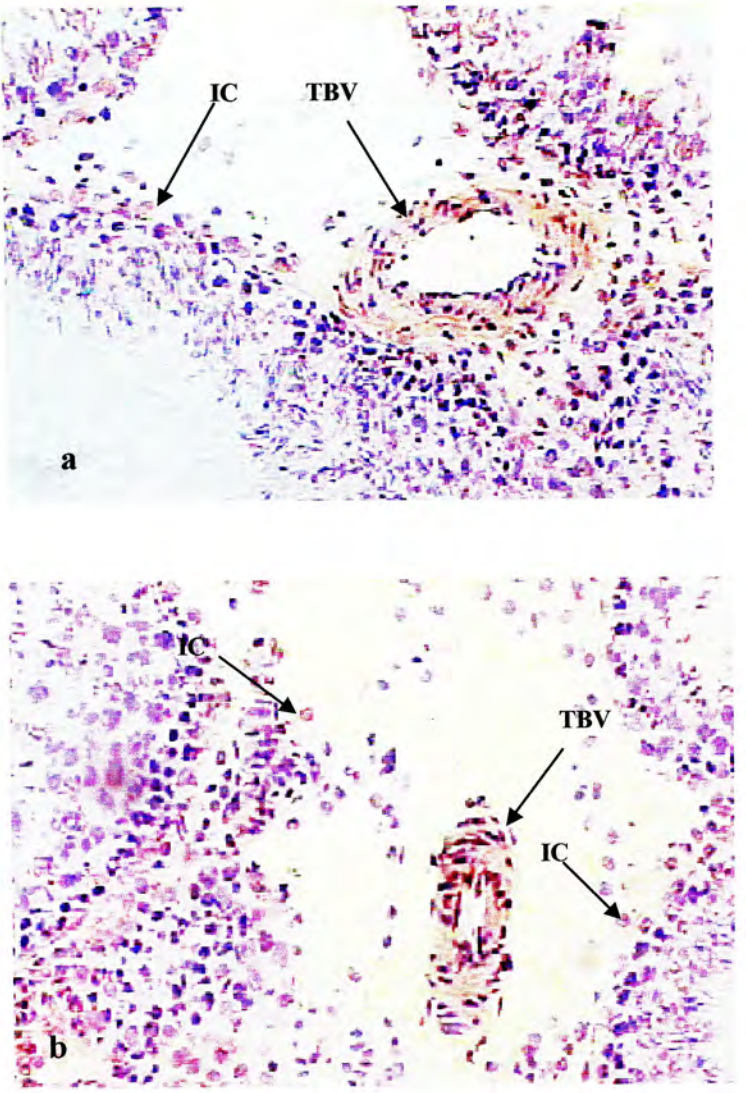


Figure 3.20 Immunohistochemical localization of Ang-3 protein on frozen sections of adult rat testes. Frozen sections were incubated with a goat polyclonal [Ang-1/4 (C-19), #sc-9360] antibody (1:100). Ang-4 immunoreactivity was predominantly localized over the vascular smooth muscle of rat testicular blood vessel (TBV). The interstitial cells found between the seminiferous tubules also exhibited weak immunoreactivity for Ang 3. Magnification x400. Sections were counterstained with haematoxylin.

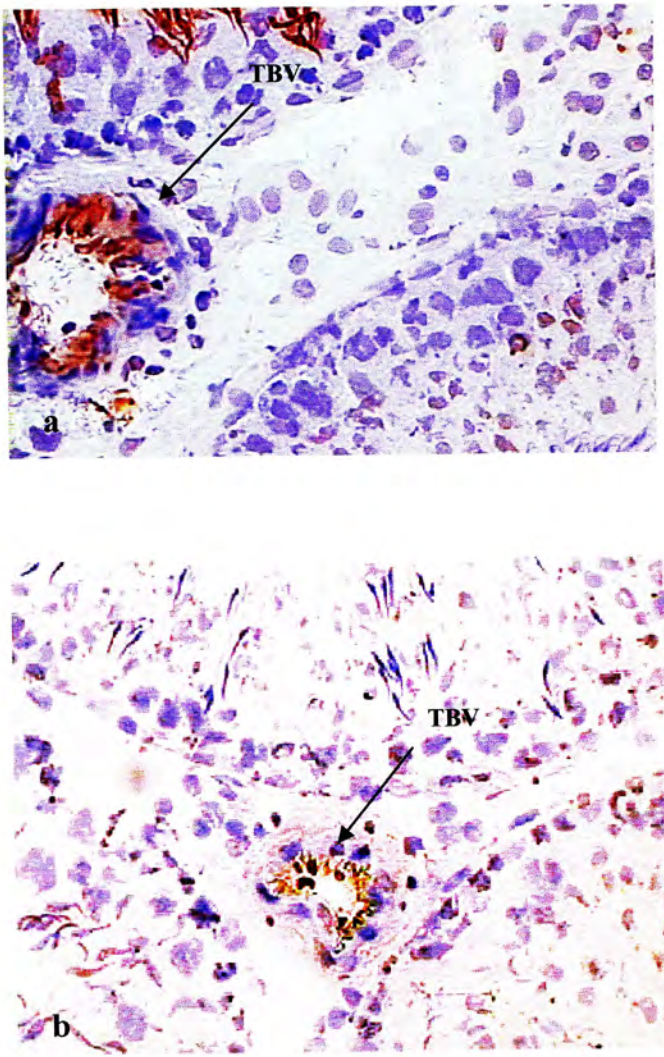


Figure 3.21 Immunohistochemical localization of Ang-1 and Ang-2 on frozen sections of adult rat testes. Frozen sections were incubated with a goat polyclonal (a) [Ang-1 (N-18), #sc-6319] and (b) [Ang-2 (C-19), #sc-7015] antibody (1:100). Red coloured areas represented localization of Ang-1 and Ang-2 over vascular smooth muscle and endothelial cells of rat testicular blood vessel (TBV) respectively. Magnification x400. Sections were counterstained with haematoxylin.

3.7 Comparison of angiopoietin expression patterns in testis using RT-PCR, Western immunoblotting and immunohistochemistry

Testis/testicular cell types/ cell lines	Nested PCR or RT-PCR	Western immunoblotting	Immuno-histochemistry
Adult rat testis	With Ang-1, Ang-2 and Ang-3 expression	With Ang-1 and Ang-2 protein expression	With Ang-1, Ang-2 and Ang-3 immunoreactivities
Mouse testis	With Ang-1, Ang-2 and Ang-3 expression	With Ang-1 and Ang-2 protein expression	-
Rat testicular blood vessel	With Ang-1, Ang-2 and Ang-3 expression	With Ang-1 and Ang-2 protein expression	With Ang-1 and Ang-3 immunoreactivities in vascular smooth muscle cells and Ang-2 immunoreactivity in vascular endothelial cells
Primary Leydig cells	With Ang-1, Ang-2 and Ang-3 expression	With Ang-1 and Ang-2 protein expression in primary interstitial cells	No Ang-1 and Ang-2 immunoreactivities, but with Ang-3 immunoreactivity in interstitial cells
Primary Sertoli cells	With Ang-1, Ang-2 and Ang-3 expression	With Ang-1 and Ang-2 protein expression	No Ang-1, Ang-2 and Ang-3 immunoreactivities
Primary germ cells	With Ang-1, Ang-2 and Ang-3 expression	No Ang-1 and Ang-2 protein expression	No Ang-1, Ang-2 and Ang-3 immunoreactivities
Mouse Leydig cell lines TM3 and MLTC-1; Rat Leydig cell lines LC540 and R2C	With Ang-1 expression in LC540 and R2C, Ang-2 expression in TM3 and Ang-3 expression in TM3, MLTC-1, LC540 and R2C	With Ang-1 protein expression in TM3, MLTC-1 and R2C and Ang-2 protein expression in MLTC-1, LC540 and R2C	-
Mouse Sertoli cell line TM4	With Ang-1, Ang-2 and Ang-3 expression	With Ang-1 protein expression, but no Ang-2 protein expression	-

3.8 Comparison of Tie 1 and Tie 2 expression patterns in testis using RT-PCR and immunohistochemistry

Testis/testicular blood vessel	RT-PCR	Immunohistochemistry
Adult rat testis	With Tie 1 and Tie 2 expression	With Tie 1 and Tie 2 immunoreactivities
Rat testicular blood vessel	With Tie 1 and Tie 2 expression	With Tie 1 and Tie 2 immunoreactivities in vascular endothelial cells

4. Discussion

Although extensive studies have been carried out trying to define the role of angiopoietins in the process of angiogenesis [Hanahan, 1997], and in particular, its interaction with VEGF, information regarding its tissue/organ distribution and regulation of expression is still scanty. This is especially true in the case of the male reproductive system which receives considerably less attention when compared with the female reproductive system [Geva and Jaffe, 2000]. The overall aim of the present investigation was therefore to study the expression of Ang-1, Ang-2, and Ang-3 in the testes since previous reports have demonstrated the expression of VEGF in the two major testicular cell types, namely Leydig cells and Sertoli cells [Collin & Bergh, 1996; Au *et al.*, 1997, Ergun *et al.*, 1997], and VEGF could be involved in hCG-induced testicular angiogenesis [Collin & Bergh, 1996] and Leydig cell-testicular macrophage interaction [Au *et al.*, 1998]. In order to localize the expression of angiopoietin to specific cell types within the testis, testicular cell lines were also used to correlate the findings collected from whole testes and preparations of primary testicular cells. The studies involved three major techniques which included RT-PCR, Western blotting, and immunohistochemistry. However as seen from the data present in the Results section and also from the following discussion, the localization of angiopoietin protein expression was hampered by the lack of highly specific antibodies that are commercially available. At the same time when the angiopoietin expression was being examined, their receptors Tie 2 and the closely related Tie 1 were also studied for their presence in the testis and in particular, the testicular blood vessel.

Angiopoietins could come from a number of cellular sources in the testis. In the present study, three mouse testicular cell lines, two rat testicular cell lines and

one rat glioma cell line were chosen for investigation since the cell lines would provide pure cell preparations which would be free from other contaminating cells. This could not be achieved even when the most stringent purification procedure was used in the preparation of primary testicular cells. Nevertheless, the major drawback with the use of the cell lines is that the phenotypic characteristics of these clonal or tumour cells may have changed during prolonged passages in culture. To partly overcome such difficulties, the same cell type (e.g. Leydig cells) derived from different origins and animal species was used to determine whether a generalized pattern of expression could emerge. The rat C6 glioma cell line was included as a positive control since previous studies indicated that it expressed both Ang-1 and Ang-2.

Both TM3 cells and TM4 cells are derived from primary culture of Leydig cell enriched preparations and Sertoli cell enriched preparations from normal testes of 11-13 day old BALB/c nu/+ mice, respectively. They exhibit characteristics similar to primary cultures of Leydig cells and Sertoli cells, and both of them are clonal cell lines. Also, their doubling times were relatively short compared with other cell lines so that RNA and proteins from these cell lines could be made available within a shorter period of time.

MLTC-1 cells are established from the M5480P transplantable Leydig cell tumour carried in C57BL/6 mice. LC540 cells are derived from a spontaneous transplantable Leydig cell tumour in a Fischer strain rat. R2C cells belong to a steriod-secreting Leydig tumour cell line derived from Wistar-Furth rats and are capable of secreting large quantities of steriod hormone even without stimulation with cAMP. These testicular cell lines have been commonly used by many investigators since they are commercially available.

Angiopoietin expression was also analysed in primary Leydig cells, Sertoli cells and germ cells using RT-PCR and Western blot analysis. The major reason for using primary testicular cells despite the problem with cell purity was that they are more likely to retain the normal characteristics of the corresponding cell types, and the information could be correlated to the ones obtained from the studies of cell lines.

Primary Sertoli cells were prepared from 20-day old immature Sprague-Dawley (SD) rats instead of adult animals mainly for two reasons. The first is that unlike the adult Sertoli cells, they retain responsiveness to FSH. The second reason is that the contamination of the Sertoli cell preparation by germ cells is less since in immature animals, the testes still do not have the full complement of germ cells.

Testicular tissues were obtained from adult Sprague-Dawley rats and ICR mice as their analyses would provide a broad picture on the expression of angiopoietin and Tie in the testis. Also they are convenient source of tissues for pilot experiments and they represent commonly used laboratory animal species.

4.1 Expression of Ang-1 mRNA and protein in adult rat testis, mouse testis, rat testicular blood vessel, primary testicular cells and testicular cell lines

Using RT-PCR, both the rat and mouse testes were shown to express Ang-1 transcripts. However when the study was extended to primary testicular cells and testicular cell lines, some samples required the use of nested PCR in order to increase the specificity of the amplification reaction. Weak signals or multiple bands were obtained when using the first set of primers (S1+AS1) alone, and this could be attributed to the design of the primers, the conditions chosen for the

amplification, the quality of mRNA preparations, or the low copy numbers of Ang-1 mRNA.

When nested PCR was applied to the analyses of all samples, it was determined that the expression of Ang-1 in adult rat testes could be associated with the Leydig cells, Sertoli cells, germ cells and testicular artery (Figure 3.1). The latter is in turn made up of mainly vascular smooth muscle and endothelial cells (Figure 3.21a). The Ang-1 expression in immature rat Sertoli cells was again observed in the study of TM4 mouse Sertoli cells (Figure 3.2). However for the demonstration of Ang-1 mRNA expression in Leydig cells, the RT-PCR results were more variable since the positive signals could be identified in LC540 and R2C rat Leydig tumour cells but were absent from TM3 and MLTC-1 mouse Leydig cells (Figure 3.2). Although this could be explained by the difference in the characteristics of these Leydig cell lines once they have been passaged in culture for many generations, however the more likely explanation was that the negative findings in TM3 and MLTC-1 cells were the results of experimental errors. First, the primer sets were able to amplify Ang-1 cDNA from the mouse testes, thus they work well for both the rat and mouse species. Second, the absence of Ang-1 transcripts in TM3 and MLTC-1 cells contradicted the presence of positive protein bands in these cell lysates when examined using Western immunoblotting. Therefore based on the RT-PCR results, it was possible to conclude that Ang-1 was expressed in the testis where it could have originated from the wall of blood vessels, Sertoli cells, Leydig cells and possibly germ cells.

The present study demonstrated the expression of Ang-1 mRNA in the testicular subcapsular artery. Also, using immunohistochemical staining, Ang-1 immunoreactivity in the testis sections was mainly localized in the vascular smooth

muscle cells around the major arteries/arterioles (Figure 3.21a). This is in agreement with earlier findings in other tissues where Ang-1 immunoreactivity was also found to be mainly localized in perivascular or vascular smooth cells, rather than endothelial cells of blood vessels. Using RT-PCR analysis, Mandriota & Pepper (1998) revealed that none of endothelial cell types (i.e. bovine microvascular endothelial cells, aortic, pulmonary artery and transformed fetal aortic endothelial cells) expressed appreciable levels of Ang-1, whereas smooth muscle cells expressed both Ang-1 and Ang-2. Using the same approach, Kim *et al.* (2000b) demonstrated the presence of Ang-1 mRNA in human umbilical vascular smooth muscle cells (HUVSMCs) but not in human umbilical vascular endothelial cells (HUVECs). Despite the fact that most studies failed to demonstrate Ang-1 expression in vascular endothelial cells, Oh *et al.* (1999) used the technique of Northern blot analysis and identified the presence of Ang-1 mRNA in both bovine retinal endothelial cells (BRECs) and bovine aortic endothelial cells (BAECs). Therefore it appeared that Ang-1 could come from both vascular smooth muscle cells (major) and endothelial cells (minor) to exert a paracrine or autocrine effect in stabilizing the vascular endothelium either by promoting cell-cell or cell-extracellular matrix interaction and/or by serving as a cell survival signal for the nonproliferating endothelial cells [Kim *et al.*, 1999a].

In the present study, Ang-1 mRNA was detectable in adult rat and mouse testes using nested PCR analysis. The authenticity of the PCR products was also confirmed by DNA sequencing and agreed well with the published cDNA sequence for rat Ang-1 (Figure 3.3).

In agreement with the demonstration of Ang-1 transcripts using RT-PCR, Western immunoblotting revealed the presence of Ang-1 protein in the adult rat

testis, Sertoli cells, Leydig cells and testicular blood vessels (Figure 3.18). Previous studies have identified two forms of Ang-1 with different molecular sizes. They represent the newly synthesized Ang-1 which is non-glycosylated and the mature product which is glycosylated [Kwak *et al.*, 1999; Kim *et al.*, 1999b]. The non-glycosylated Ang-1 is predominantly found in cell lysates while the glycosylated form is usually secreted from cells and found in the medium of cultured cells [Kwak *et al.*, 1999; Kim *et al.*, 1999b]. In line with these earlier findings, the present study also demonstrated the occurrence of two positive bands with apparent molecular sizes of 58 kDa and 72 kDa. This could be clearly seen in the lysates prepared from adult rat testis, mouse testis, testicular blood vessel, testicular interstitial cells, primary Sertoli cells, all testicular cell lines (with the exception of LC540), and C6 rat glioma cells which was used as a positive control (Figures 3.17 and 3.18). In these samples, the lower molecular size species representing the non-glycosylated form invariably gave a stronger signal than the high molecular weight glycosylated form, again pointing to the predominance of newly synthesized, non-glycosylated Ang-1 inside the cells. In LC540 cells, only the smaller molecular size protein band could be seen, and this could be explained by a low abundance of Ang-1 relative to the same amount of total proteins used for Western immunoblotting. In the germ cells, no protein bands of the correct sizes could be identified (Figure 3.18). Thus it remained unclear whether in this cell type, the presence of Ang-1 transcripts as revealed by RT-PCR represented the contribution from neighbouring Sertoli cells or the Ang-1 protein was processed differently in this mixed population of haploid (i.e. spermatids) and diploid cells (i.e. spermatogonia and spermatocytes).

Detailed examination of the Western blots revealed that not only the lower molecular size band gave stronger signal, it might also encompass another protein band with a slightly lower molecular size (~55 kDa). As it would be discussed in the next section, it might represent the cross-binding of this particular antibody to Ang-2, which has a slightly smaller molecular weight than Ang-1 [Kim *et al.*, 2000a].

In the present investigation, it was not possible to demonstrate the presence of alternatively spliced Ang-1 mRNA in the adult rat testis and other testicular cell lines by RT-PCR (Figures 3.4 and 3.5). Since the original work was done in human, it does not necessary mean that this could be generalized to other species such as the rat and mouse. Even in the original publication [Huang *et al.*, 2000], it could be seen that these alternatively spliced species had very limited distribution occurring only in a few human cell lines and fetal cells. In the cells where these spliced variants of Ang-1 mRNA were found, most of them only expressed two to three but not all four alternatively spliced species. Unfortunately in the present study, it was not possible to acquire these human cells to serve as positive controls, thus the observed negative findings might not be definitive.

When the results obtained by RT-PCR and Western immunoblotting were compared with those by immunohistochemical staining, there appeared to be a discrepancy. Using the first two techniques, both Sertoli cells and Leydig cells were shown to express Ang-1 mRNA (Figures 3.1 and 3.2) and protein (Figures 3.17 and 3.18). However when frozen sections of adult rat testis were immunostained for Ang-1 using specific antibody, positive Ang-1 immunoreactivity was localized only over the blood vessels mainly in vascular smooth muscle cells (Figure 3.21a). Such findings were not entirely unexpected since the antibody used

for Western immunoblotting (Ang-1, H-98) was different from the one employed in immunohistochemcial staining (Ang-1, N-18). It is known that some antibodies are suitable only for Western immunoblotting (e.g. Ang-1, H-98) since they only recognize the denatured form but not the native form of the protein. Also in immunostaining, the ability of the antibody to recognize the antigen depends very much how well the antigen is preserved. It was possible that in frozen testis sections subjected to light fixation in paraformaldehyde, the Ang-1 protein was for some unknown reasons better preserved in blood vessels than in the Sertoli cells and Leydig cells (Figure 3.21a). In the present study, other fixatives had been tested together with the technique of antigen retrieval in the preparation of the testis sections for immunostaining (data not shown), however none of them gave more satisfactory results than the use of lightly fixed frozen sections.

The expression of Ang-1 mRNA in rat C6 glioma cells was previously demonstrated in other studies by means of RT-PCR, Northern blot analyses and *in situ* hybridization [Holash *et al.*, 1999]. The ability to reproduce the findings here in demonstrating the presence of both Ang-1 mRNA and proteins using RT-PCR (Figures 3.1 and 3.2) and Western immunoblotting (Figures 3.17 and 3.18), respectively, had further added strength to the validity of the present results.

4.2 Expression of Ang-2 mRNA and protein in adult rat testis, mouse testis, rat testicular blood vessel, primary testicular cells and testicular cell lines

There has been much recent interest in the role of Ang-2 at sites of vascular remodeling in an otherwise stable adult vasculature [Mandriota & Pepper, 1998; Maisonpierre *et* al., 1999; Holash *et al.*, 1999; see review by Geva & Jaffe, 2000]. Restricted expression pattern of Ang-2 was observed in ovary, uterus, placenta and

endothelial cells, which were the predominant sites of vascular remodeling in the normal adult [Hanahan, 1997; Maisonpierre *et al.*, 1997; Shyu *et al.*, 1998; Shinji *et al.*, 1999; Kämpfer *et al.*, 2001], and the observation of cell type-specific expression of both angiopoietins [Stratmann *et al.*, 2001].

Similar to the expression pattern of Ang-1 mRNA within the testis, the present data also showed the expression of Ang-2 mRNA in adult rat and mouse testes, Leydig cells, Sertoli cells, germ cells, and rat testicular blood vessel (Figures 3.6 and 3.7). However it was not possible to compare the expression levels of Ang-1 and Ang-2 mRNA in different testicular tissues and cell types since the PCR analysis was not performed in a quantitative manner.

In the RT-PCR analysis of testicular cell lines, the expression of Ang-2 appeared to be more restricted and found only in TM3 Leydig cells and TM4 Sertoli cells (Figure 3.7). Surprisingly the three Leydig tumour cell lines (i.e. MLTC-1, LC540, R2C) were negative for Ang-2. Contrary to the present findings, previous studies indicate that tumour cells are more likely to express Ang-2 and this appears to tie in with the postulated role of Ang-2 in promoting angiogenesis (including tumour angiogenesis) in the presence of VEGF (Introduction: Section 1.3.2.). Ang-2 expression had in the past been reported in human gastric tumour cells [Etoh *et al.*, 2001], tumour cells of Kaposi's sarcoma and cutaneous angiosarcoma [Brown *et al.*, 2000] and human hepatocellular carcinoma [Tanaka *et al.*, 1999]. The inability to demonstrate Ang-2 mRNA expression in Leydig tumour cells remains unclear since in Western immunoblotting, there appears to be protein bands in the cell lysates of MLTC-1, LC540 and R2C cells, that could represent Ang-2 (Figure 3.17). In the Western immunoblotting of Ang-1 using an antibody that cross-react with Ang-2, a protein band with a slightly lower molecular size

(~55 kDa) than expected for the non-glycosylated form of Ang-1 (~58 kDa) was found in the rat testis, and testicular cell lines (i.e. TM3, MLTC-1, R2C and LC540) (Figure 3.17). According to the calculated molecular weight of nascent proteins, Ang-2 (56.4 kDa) is slightly smaller than Ang-1 (57.4 kDa), suggesting that the smaller band might represent Ang-2 due to the cross-reactivity of this particular antibody [Ang-1 (H-98)]. In the subsequent Western blot analysis using an antibody specific for Ang-2, two positive bands with apparent molecular sizes of 55 kDa and 68 kDa could be identified in the lysates prepared from the testicular tissues (i.e. rat and mouse testes, testicular blood vessels), primary testicular cells (i.e. interstitial cells and Sertoli cells), and C6 rat glioma cells which was used as a positive control. However at this stage, the above study using the specific antibody against Ang-2 has not been successfully repeated on the testicular cell lines to confirm whether the Leydig tumour cells (i.e. MLTC-1, R2C and LC540) are in fact producing Ang-2 proteins despite low to undetectable levels of mRNA (as discussed above)

Consistent with the expression of Ang-2 mRNA in adult rat testis, mouse testis, primary Sertoli cells and Leydig cells, Western immunoblotting demonstrated the occurrence of two positive bands with apparent molecular sizes of 55 kDa and 68 kDa (Figure 3.19). It is likely that they correspond to the non-glycosylated and glycosylated form of Ang-2 since previous studies of human Ang-2 have cited 56.9 kDa [Kim *et al.*, 2000a] and 68-70 kDa [Dunk *et al.*, 2000; Kuroda *et al.*, 2001], respectively for the native (i.e. non-glycosylated) and secreted (i.e. glycosylated) form of this protein.

Though in RT-PCR analysis, germ cells were shown to express Ang-2 mRNA, no protein bands of the correct sizes could be identified in their lysates in Western

blot analysis. Thus it is possible that Ang-2 transcripts found in germ cell preparation could have derived from the contamination of other neighbouring testicular cells (e.g. Sertoli cells) truly expressing this gene.

In determining whether primary testicular cells express Ang-2 protein, the data from immunohistochemical staining (Figure 3.21b) failed to provide the needed information. In frozen sections of the adult rat testis, the failure to demonstrate Ang-2 immunoreactivity in Sertoli cells and Leydig cells could be due to the fact that the conditions of immunostaining had not been fully optimized (Figure 3.21b). Nevertheless, it would still be tempting to suggest that Ang-2 mRNA and proteins are expressed in the Sertoli cell and Leydig cell of the rat and mouse testes, where Ang-1 was also found (Figure 3.18). In the human placenta, Ang-2 mRNA expression was identified in syncytiotrophoblasts [Goldman-Wohl *et al.*, 2000], which are also endocrine cells like Sertoli cells and Leydig cells.

The present finding of Ang-2 mRNA expression in testicular blood vessel agrees well with the previous reports on Ang-2 expression in vascular smooth muscle cells [Yuan *et al.*, 2000] as well as vascular endothelial cells [Mandriota & Pepper, 1998; Stratmann *et al.*, 1998; Oh *et al.*, 1999; Kim *et al.*, 2000a; Kim *et al.*, 2000c; Kuroda *et al.*, 2001]. However in immunohistochemical staining, Ang-2 immunoreactivity was only found to localized in endothelial cells but not vascular smooth muscle cells of testicular blood vessels (Figure 3.21b).

Earlier studies reported that VEGF was expressed in Leydig and Sertoli cells of rat, mouse and human testes (as mentioned in Section 4.1). These testicular cell types would be the important sources of angiogenic factors during the development of the testis and/or following hormonal stimulation as seen in adult rats after receiving a single injection of LH/hCG [Schultz *et al.*, 1995; Au *et al.*, 1996; Collin

& Bergh, 1996; Bergh *et al.*, 1996]. Taken together, these observations are in line with the proposed function of Ang-2, in which its expression facilitates angiogenesis in the presence of VEGF. Ang-2 works by blocking a constitutive stabilizing or maturing function for Ang-1 on blood vessels. Such Ang-2 negative signal would thus prime the blood vessels and facilitate their responsiveness towards other angiogenic inducers such as VEGF by loosening contacts between endothelium and peri-endothelial cells [Hanahan, 1997; Maisonpierre *et al.*, 1997; Holash *et al.*, 1999; see review by Yancopoulos *et al.*, 2000].

Unlike the situation found in chicken testes [Mezquita *et al.*, 1999, 2000], human endothelial cells, some human nonendothelial tumour cell lines and tissues [Kim *et al.*, 2000a] where alternatively spliced variants of Ang-2 were identified, the present study failed to demonstrate any variants of Ang-2 in the rat testis that might resemble the splicing found in human (Figure 3.9). A study performed by Kim *et al.* (2000a) has led to the discovery of a novel alternative splice variant of the human Ang-2 gene designated as Ang-2_{443} in primary endothelial cells, several nonendothelial tumour cell lines, and primary tumour tissues based on cloning and cDNA analysis. The present study however failed to demonstrate any Ang-2 isoform expression that could be detected in the adult rat testis using RT-PCR approach with degenerate Ang-2 (S3+AS4) primers even after a second PCR amplification (Figure 3.9). The PCR products were confirmed negative by automated DNA sequence analysis. It is possible that alternatively splicing to generate multiple species of Ang-2 is species specific as demonstrated in human and chicken.

In a previous study, it was unable to detect Ang-2 mRNA transcripts by RT-PCR analysis in C6 glioma cells [Mandriota & Pepper, 1998]. Contrary to this

earlier report, the present study showed the presence of Ang-2 mRNA in C6 rat glioma cells (Figures 3.6 and 3.7), suggesting its biologic importance in tumour-associated angiogenesis. It is possible that the negative findings in the study by Mandriota & Pepper (1998) was due to the use of degenerate primers.

4.3 Expression of Ang-3 mRNA and protein in adult rat testis, mouse testis, rat testicular blood vessel, primary testicular cells and testicular cell lines

The present study provided the first demonstration of Ang-3 mRNA expression in rat testicular tissue. In the study by Valenzuela *et al.* (1999) reporting on the identification of Ang-3 in mouse and Ang-4 in human, the expression of Ang-3 mRNA in mouse testicular tissue was demonstrated using Northern blot analysis. In line with this earlier report, the present study found an apparently higher level of Ang-3 mRNA expression in mouse testes and TM4 testicular cells (Figures 3.10 and 3.11). Nevertheless, Ang-3 mRNA was also detectable in rat testes, testicular blood vessel, primary Sertoli cells, Leydig cells, germ cells (Figure 3.10). As for the other testicular cells (besides TM4) and C6 glioma cells, Ang-3 transcripts were still detectable in rat (LC540 and R2C) and mouse (TM3 and MLTC-1) Leydig cell lines (Figure 3.11). Therefore, Leydig cells, Sertoli cells and germ cells are the possible sources of Ang-3 in the testis. Valenzuela *et al.* (1999) proposed the role of Ang-3 in mouse and Ang-4 in human. Ang-3 and Ang-4 represent the mouse and human counterparts of the same gene locus and their structural divergence appears to underlie diverging functions of these counterparts. While human Ang-4 is an agonist for the Tie 2 receptor, Ang-3 appears to act as an antagonist (as reviewed in the Introduction). Nevertheless, the exact functional

role of Ang-3 in testis remains not clearly defined but the current findings would encourage further studies to be carried out.

The identity of the Ang-3 product band amplified from adult rat testis had also been confirmed by automated DNA sequencing. Alignment of the known nucleotide sequence of mouse Ang-3 with the nucleotide sequence of PCR product amplified from adult rat testis indicated that the nucleotide sequences of mouse Ang-3 and rat Ang-3 are highly conserved (Figure 3.12). The present study represented the first to report on the nucleotide sequence of Ang-3 in the rat species, though the coverage was partial and only spanned over 543 bp.

The lack of suitable antibodies for Western blotting precluded the correlation between Ang-3 mRNA and protein expression in testicular tissues, various primary testicular cells and testicular cell lines. With the commercially available anti-Ang-3 antibodies, they were found to be relatively non-specific. As for another antibody which cross-reacts with Ang-1, mouse Ang-3 and human Ang-4, it was tested for immunohistochemical staining and found to give positive immunoreactivity over the vascular smooth muscle cells of testicular blood vessels and interstitial cells (Figures 3.20a and 3.20b). Since this antibody may cross-react with Ang-1, the results thus generated on its use to localize Ang-3 protein in rat testis sections have to be interpreted with great caution. At least, the positive Ang-3 immunoreactivity associated with testicular blood vessels and interstitial cells (Figures 3.20a and 3.20b) would not contradict with the expression of Ang-3 transcript in rat testicular blood vessel and Leydig cells (Figure 3.10). Further investigation will be required to explore the reasons why testicular interstitial cells with stationary vasculature are still able to secrete angiogenic factors and the roles of both Ang-3 and Ang-4 in vascular development.

4.4 Expression of Tie 1 and Tie 2 mRNAs and proteins in adult rat testis and rat testicular blood vessel

Tie 1 and Tie 2 are known to be endothelial cell-specific receptor tyrosine kinase. In the present study, use of RT-PCR demonstrated their mRNA expression in the adult rat testis and testicular blood vessels (Figures 3.13 and 3.14). However, it would be better to include an endothelial cell line as a positive control for RT-PCR analysis and compare the expression of Tie receptor kinases between rat testicular blood vessel and this endothelial cell line. Using immunohistochemical staining, both Tie 1 and Tie 2 immunoreactivities were localized to endothelial cells of testicular blood vessels (Figures 3.19a and 3.19b). Thus the endothelial cells lining the testicular vasculature contain receptors (i.e. Tie 2) for angiopoietins, allowing them to mediate the actions of angiopoietins (e.g. Ang-1, -2 and -3) or other unidentified ligand for Tie 1 in controlling the integrity and remodelling of the testicular blood vessels. Such co-expression patterns have also been observed in other tissues, including the endothelium of large and small blood vessels, the atrial and ventricular endothelia of a 15-week human fetal heart [Partanen *et al.*, 1999] as well as in blood vessels, synovial lining and stromal cells in all rheumatoid arthritis (RA) tissues [Uchida *et al.*, 2000]. A possible mechanism underlying such observed apparent co-expression of Tie 1 and Tie 2 is the combinations of ligands or ligand heterodimers for induction of endothelial responses by coengaging the two Tie receptors [Maisonpierre *et al.*, 1993].

Recent studies have also identified the presence of Tie 1 and Tie 2 receptors in other non-endothelial cell types such as glandular epithelium of human endometrium (for Tie 2) [Geva & Jaffe, 2000], trophoblast of human placenta (for Tie2) [Dunk *et al.*, 2000; Goldman-Wohl *et al.*, 2000], pigmented epithelial and

stromal cells of the retina [Otani *et al.*, 1999], synovial lining cells and stromal cells of rheumatoid arthritis tissues [Uchida *et al.*, 2000], and tumour cells of Kaposi's sarcoma and cutaneous angiosarcoma [Brown *et al.*, 2000]. Therefore future works are required to examine whether Tie 1 and 2 could also be found in other testicular cell types. If such evidence could be found, it would offer the possibility of additional non-endothelial effects of angiopoietins on other aspects of testicular function under physiological or pathophysiological states.

In the present study, Tie 1 and Tie 2 mRNA transcripts were detectable in adult rat testes and testicular blood vessels using RT-PCR analysis. The identity of the Tie 1 product bands amplified from adult rat testis and rat testicular blood vessel had also been confirmed by automated DNA sequencing. However, it was not possible to compare the nucleotide sequences of these PCR products directly with published sequence of rat Tie 1, but alignment of the known nucleotide sequence of mouse Tie 1 with the nucleotide sequences of PCR products amplified from adult rat testis and rat testicular blood vessel indicated that the nucleotide sequences of mouse Tie 1 and rat Tie 1 are highly conserved (Figure 3.15). The authenticity of the Tie 2 product band amplified from rat testicular blood vessel was also confirmed by DNA sequencing and agreed well with the published cDNA sequence for rat Tie 2 (Figure 3.16).

The above-mentioned testicular cell lines and primary testicular cells were used for RT-PCR and Western blot analysis in the current investigation. The present data clearly showed the expression of Ang-1, Ang-2 and Ang-3 in Sertoli cells, Leydig cells, germ cells, rat testicular blood vessel, adult rat testis and mouse testis, as well as the expression of Tie 1 and Tie 2 in rat testicular blood vessel and adult rat testis using RT-PCR analysis. Because of this striking similarity in

molecular structure, molecular weights and sequence homology between Ang-1 and Ang-2 proteins, Western immunoblotting for Ang-1 and Ang-2 was performed with a rabbit polyclonal anti-Ang-1 antibody [Hanahan, 1997]. Since this rabbit polyclonal antibody [Ang-1 (H-98), #sc-8357] used in this study exhibited cross-reactivity with Ang-2 and both Ang-1 and Ang-2 were reported to be secreted glycoproteins of the same molecular weight of 75 kDa approximately as described previously, it was impossible to distinguish which positive protein bands corresponded to either Ang-1 or Ang-2 for each sample and the expression of Ang-1 could not be examined by immunoblot as the commercially available anti-Ang-1 antibodies were not suitable for this application. The non-glycosylated and glycosylated forms of Ang-1 and Ang-2 proteins are found to express in Sertoli cells, interstitial cells, germ cells, rat testicular blood vessel and adult rat testis. Immunohistochemical examination of the rat testes using specific antibodies raised against Ang-1, Ang-2, Ang-3, Tie 1 and Tie 2 provided evidence for the localization of angiopoietins, Tie 1 and Tie 2 proteins in rat testicular blood vessel.

4.5 Conclusion

Taken together, both Leydig and Sertoli cells of rat and mouse testis are possible sources of Ang-1, -2, and -3. This would be analogous to the situation found in the ovary where granulosa cells of pre-ovulatory follicles were shown to express Ang-1 mRNA [Hazzard *et al.*, 1999] and possibly also Ang-2 [Masionpierre *et al.*, 1997; Geva & Jaffe, 2000]. In view of the fact that both Leydig cell and Sertoli cell are important sources of VEGF within the testis [Collin & Bergh, 1996; Au *et al.*, 1997; Ergun *et al.*, 1997], therefore they appear to hold a

pivotal role in determining the integrity and angiogenic potential of the testicular vasculature through coordinated changes in the expression of those factors that are responsible for stabilizing the blood vessels (i.e. Ang-1) or for angiogenesis (i.e. VEGF, Ang-2).

4.6 Further work

In the present study, the mRNA expression of angiopoietins (Ang-1, Ang-2 and Ang-3) and Tie receptor tyrosine kinases has been demonstrated by RT-PCR in the testis. In addition, the protein expression of angiopoietins, Tie 1 and Tie 2 have been demonstrated by Western blot analysis and localized in adult rat testis by immunohistochemistry. It is therefore of special interest in investigating the control of angiopoietin expression in the testes using the combination of RT-PCR and capillary electrophoresis with laser-induced fluorescence detector (CE-LIF) for accurate quantitation of mRNA levels of angiopoietins in testicular tissue or on *in vitro* cultures of testicular cell lines within the linear range of PCR cycle-product curve and PCR template-product curves. The PCR amplification efficiencies of angiopoietins and the reference gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or β-actin are determined according to these PCR-cycle product curve and PCR template-product curve.

As the expression of Ang-2 mRNA has been shown in both TM3 and TM4 testicular cell lines by RT-PCR and Oh *et al.* (1999) suggested that hypoxia probably produced a direct stimulation of Ang-2 mRNA levels in BRECs (as explained earlier in Section 1.3.2), the effect of hypoxia on Ang-2 expression in TM3 and TM4 cells is the next interesting issue to deal with. Similar to this

published study, an up-regulation of Ang-2 expression should be expected in both TM3 Leydig cells and TM4 Sertoli cells. Such *in vitro* findings of a hypoxic induction of Ang-2 expression in Leydig cells and Sertoli cells can be correlated with an *in vivo* situation. This can be done by investigating the effect of the ischaemic condition produced by testicular torsion in adult rats. Similar to *in vitro* findings, Ang-2 mRNA levels should also be increased in those testes that had been exposed to an ischaemic treatment caused by the surgical induction of spermatic cord torsion.

The hormonal regulation of Ang-2 expression in the testis should be the next component for studying in order to provide useful information of the hormonal effects on the developmental changes of rat testis. MLTC-1 cells derived from Leydig cell tumour are known to retain their LH responsiveness even after repeated passages in culture rather than TM3 and TM4 testicular cells. In this regard, MLTC-1 testicular cells should be employed for studying the effects of hCG treatment on the expression of Ang-2 mRNA in these cells. After hCG treatment, Ang-2 mRNA expression should be increased significantly in MLTC-1 testicular cells and such stimulatory effects should be both time- and concentration-dependent. Ang-2 mRNA levels can also be determined in the testes of adult rats after receiving a single subcutaneous injection of hCG in order to correlate with *in vitro* findings using MLTC-1 Leydig cell line.

Huang *et al.* (2000) has previously demonstrated the existence of two Ang-1 isoforms of Ang-1, Ang-1.5 kb and Ang-0.9 kb, in CHRF-288 human tumour cell line and prostate cancer cell line DU145, rather than Ang-1.3 kb and Ang-0.7 kb using Western immunoblot analysis with a specific rabbit anti-Ang-1 antibody. In the current study, no alternatively spliced species of Ang-1 mRNA was detected in

adult rat testis and other testicular cell types using RT-PCR approach with specific primers (as mentioned in Section 4.1). Further investigations were necessary to confirm whether the protein isoforms of Ang-1 could be expressed in testis and other testicular cell types or not by Western blot analysis with an anti-Ang-1 antibody specific for the N-terminal end (reactive with anti-1.5 kb and anti-1.3 kb isoforms) or C-terminal end (reactive with 1.5-kb isoform but not 1.3-kb isoform) and a goat anti-Ang-1 antibody specific for the N-terminal end (reactive with anti-0.9 kb and anti-0.7 kb isoforms) or C-terminal end (reactive with 0.9-kb isoform but not 0.7-kb isoform).

5. Reference

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